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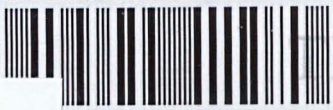
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<http://dx.doi.org/doi:10.21954/ou.ro.0000d5c6>

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The Role of UBZ1 in PCNA Dependent DNA Damage Response

Martina Colombin

A Thesis Submitted in Fulfilment of the Requirements of the
Faculty of Life Science of the Open University (UK) for the Degree
of Doctor of Philosophy



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September 2013

DATE OF SUBMISSION : 2 SEPTEMBER 2013

DATE OF AWARD : 14 NOVEMBER 2013

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ABBREVIATIONS

(6-4) (TT 6-4 PP) Thymine-Thymine Pyrimidine-Pyrimidone (6-4)

AMPK AMP Activated Protein Kinase

APC Anaphase Promoting Complex

APE1 APurinic/APrimidinic site Endonuclease 1

APIM ALKB homologue 2 PCNA Interacting Motif

ARF Alternative Reading Frame

ATM Ataxia Telangiectasia Mutated kinase

ATR Ataxia Telangiectasia and Rad3-related

ATRIP ATR-Interacting Protein

BER Base Excision Repair

BLM BLoOM syndrome protein

BP-G Benzo(a)pyrene adducts

BRCA1 BReast CAncer type 1

BRCA2 BReast CAncer type 2

CAK Cdk Activating Kinase

CAND1 Cullin Associated and Neddylation Dissociated 1

CDK Cyclin Dependent Kinase

cisPt-GG Cisplatin GG adduct

CK2 Casein Kinase 2

CPDs Cyclobutane Pyrimidine Dimers

CS Cockayne Syndrome

CTiP CtBP-Interacting Protein

DDB DNA Damage Binding

DNA PKcs DNA-dependent Protein Kinase Catalytic Subunit

DNA-PK DNA-dependent Protein Kinase

dRP DeoxyRibose Phosphate

DSB Double Strand Break

DSBR Double Strand Break Repair

DTAP Denaturing Tandem Affinity Purification

DUBs De-UBiquitinating enzymes

E6AP E6 Associated Protein

ECO1 Establishment of COhesion protein 1

EME1 Essential Meiotic Endonuclease 1

ERAD Endoplasmic Reticulum Associated Degradation

ERCC Excision Repair Cross Complementing

EXO1 EXOnuclease 1

FEN-1 Flap structure specific EndoNuclease 1

GG di-Glycine

GGR Global Genome Repair

HA HemAgglutinin

hABH2 Human AlkB Homologue 2

HAUSP Herpesvirus Associated Ubiquitin Specific Protease

HECT Homologous to the E6-AP Carboxyl Terminus

HHR23B Human Rad23 Homologue B

HMGB1 High Mobility Group Box 1

HMGN1 High Mobility Group Nucleosome 1

HR Homologous Recombination repair

IKK IκB Kinase

IR Ionizing Radiation

LC/MS Liquid Chromatography/Mass Spectrometry

LIG I DNA Ligase I

LP Long Patch

LUBAC Linear UBiquitin chain Assembly Complex

MAT Metal Affinity Tag

MAT1 Menage A Trois 1

MDC1 Mediator of DNA Damage Checkpoint protein 1

MGS1 Maintaining Genome Stability 1

MMR MisMatch Repair

MRN MRE11/RAD50/NBS1

MRX MRE11/RAD50/XRS2

NEMO NF kappa B Essential MOdulator

NER Nucleotide Excision Repair

NHEJ Non Homologous End Joining repair

NZF Npl4 Zinc Finger domain

OGG1 8-OxoGuanine-DNA Glycosylase

PARI PCNA Associated Recombination Inhibitor

PARP-1 Poly (ADP-Ribose) Polymerase I

PCNA Proliferating Cell Nuclear Antigen

PIP PCNA Interacting Peptide

PNK PolyNucleotide Kinase

PNKP PolyNucleotide Kinase/Phosphatase

POL IV DNA polymerase IV

POL λ Polymerase λ

POL μ Polymerase μ

PUA β -Unsaturated Aldehyde dehydration Product

RAD23 Radiation Sensitive 23

RAP80 Receptor Associated Protein 80

RBX Ring BoX

RFC Replication Factor C

RING Really Interesting New Gene E3

RIP Receptor Interacting Protein

RNAPII RNA Polymerase II

ROC Ras Of Complex

ROS Reactive Oxygen species

RPA Replication Protein A

SAE2 SUMO-Activating Enzyme subunit 2

SCF Skp1 Cullin F box protein

SDSA Synthesis Dependent Strand Annealing

SGS1 Small Growth Suppressor 1

SKP1 S-phase Kinase associated Protein 1

SNM1A Sensitive to Nitrogen Mustard 1A

SP Short Patch

SSR Single Strand Repair

STSs Suppressor of T cell receptor Signaling

TCR Transcription Coupled Repair

TDG Thymine DNA Glycosylase

TFIIH Transcription Factor II H

TLS TransLesion Synthesis

TNFR1 Tumor Necrosis Factor Receptor 1

TNF α Tumor Necrosis Factor α

TOPOIII α Topoisomerase III α

TRAF2 TNF Receptor Associated Factor 2

TT 6-4 PP Thymine-Thymine Pyrimidine-Pyrimidone (6-4)

UB Ubiquitin

UBA UBiquitin Associated domain

UBC UBiquitin Conjugating enzyme

UBD Ubiquitin Binding Domain

UBL UBiquitin Like domain

UBM Ubiquitin Binding Motif

UBZ Ubiquitin Binding Zinc finger

UCH-L1 Ubiquitin Carboxy-terminal Hydrolase L1

UDG Uracil-DNA Glycosylase

UEV Ubiquitin E2 Variant

UIM Ubiquitin Interacting Motif domain

USP1 Ubiquitin Specific Peptidase 1

WHIP Werner Helicase Interacting Protein

WRN Werner helicase

XAB2 XPA Binding protein 2

XLFXRCC4-Like Factor

XP Xeroderma Pigmentosum

XPA Xeroderma Pigmentosum group A

XPB Xeroderma Pigmentosum group B

XPV Xeroderma Pigmentosum Variant

XRCC4 X-ray Repair Cross-Complementing protein 1

ABSTRACT

Organisms frequently must deal with several sources of damage that can alter the cell's genomic material. DNA damage can be caused by exogenous agents, like radiation and toxins or by endogenous factors, like Reactive Oxygen Species (ROS), produced during normal oxygen metabolism. In order to guarantee cell survival and the transmission of the correct genetic information to their offspring, organisms have evolved responses to counteract the effects of DNA damage, which include for example, repair pathways and DNA damage tolerance pathways.

In this thesis, I have focused on the poorly characterized UBZ-containing protein, called Ubz1. Ubz1 contains an Ubiquitin Binding Zinc finger (UBZ) domain, which is only found in other proteins involved in DNA repair, such as polymerase η and κ , Rad18 and WHIP. Moreover, Ubz1 has a PCNA Interacting Peptide (PIP), which is important for its interaction with the Proliferating Cell Nuclear Antigen (PCNA). Besides these two domains, a third domain known as the SprT domain, which is of unknown function, is also been found in Ubz1. Currently no other eukaryotic SprT domain containing protein has been characterized. The Werner Helicase Interacting Protein (WHIP), which also has a UBZ domain, is subjected to a UBZ domain-dependent covalent ubiquitination, known as coupled ubiquitination; thus, at the beginning of my work, I performed Denaturing Tandem Affinity Purifications (DTAP), in order to verify if Ubz1 is also subjected to this post-translational modification. I found that Ubz1 does undergo coupled ubiquitination, and this occurs at four sites of ubiquitination. Furthermore, I demonstrated that Ubz1 interacts with PCNA and that mutating the UBZ domain does not disrupt binding to PCNA. However this mutant does not bind to modified PCNA. Additionally, to verify the role of the third uncharacterized SprT domain of Ubz1, I constructed the SprT domain mutant of Ubz1, and I found that it lost the ability to interact with modified or unmodified

PCNA. To further characterize the Ubz1/PCNA interaction, I generated three constructs expressing PCNA either alone or fused to a mono or tetra- ubiquitin moieties.

I found that UBZ domain of Ubz1 protects the ubiquitin bound to K164 of PCNA from being de-ubiquitinated by the SprT domain. In fact, I showed that SprT domain acts as a metalloprotease, removing the ubiquitins bound to the PCNA. Moreover, the Sprt domain mediates the Ubz1 autocleavage, which strengthens the hypothesis that it might have a metalloprotease activity.

1. INTRODUCTION

SECTION A: DNA Repair

A main feature for cell survival is the maintenance of genomic integrity. However, many DNA modifications occur daily (Table 1.1). As a matter of fact, the genome is constantly subjected to several kinds of endogenous damage. Some examples are the Reactive Oxygen Species (ROS), produced during the aerobic metabolism, the deamination of cytosines, which produces uracils, and also the incorporation of wrong nucleotides during the replication (Waters et al., 2009). Many environmental agents like UV light, ionizing radiation, and chemicals such as adducts produced by tobacco smoke or generated by chemotherapeutic drugs, can lead to mutations, genomic rearrangements and instability.

DNA damage is particularly dangerous during DNA replication, since this can lead to the collapse of the replication fork and even to the formation of double strand breaks. In addition, if the DNA damage is not correctly repaired, the accumulation of the lesions and the resulting genetic instability can eventually lead to tumorigenesis or to the death of the cell.

The cellular response to DNA damage may involve activation of cell cycle checkpoints, DNA repair pathways and if the damage is severe, can lead to apoptosis. DNA repair processes are highly conserved from bacteria to mammals and during evolution, different DNA repair pathways have evolved, because a single pathway cannot handle all the different types of DNA damage that can occur. In mammals, the most important DNA damage repair pathways are the Nucleotide Excision Repair pathway (NER), the Base Excision Repair pathway (BER), the MisMatch Repair pathway (MMR), the Non Homologous End Joining pathway (NHEJ) and the Homologous Recombination

pathway (HR) (Table 1.1). In addition, cells have evolved a process, known as Translesion Synthesis (TLS) that allows them to replicate DNA past the site of the damaged DNA (Waters et al., 2009).

DNA DAMAGE	CAUSES	REPAIR	PATHOLOGY
DSB	IR, ROS, VDJ	HR/NHEJ	Bloom syndromes
Incorrect base	DNA replication	MMR	Lynch syndromes
Deamination, oxidation, alkylation	Normal cellular metabolism	BER	Cancer?
Bulky lesions	UV/ chemicals	NER	XP/Cocaine syndromes

Table 1.1. Examples of DNA damages and repairs.

1.1. Double Strand Break Repair

Double Strand Breaks (DSBs) are generated by several natural endogenous events or exogenous agents. ROS produced by cellular respiration can cause the collapse of the replication forks, leading to DSBs. V(D)J recombination and meiosis can also lead to DSBs (Lieber et al., 2010; Shrivastav et al., 2008). DSBs can also occur when the replication machinery encounters unrepaired lesions and the replication fork collapses (Saleh-Gohari et al., 2005). External agents, such as Ionizing Radiation (IR), chemical agents, and UV light can produce DSB. Chemotherapeutic drugs such etoposide, which is a cancer-treatment drug, can also induce strand breaks in cellular DNA by trapping topoisomerase II at the site of DNA re-ligation (Hande, 1998; Shrivastav et al., 2008).

Two types of DSB repair have been discovered. These are known as Non

Homologous End Joining (NHEJ) and Homologous Recombination (HR) (Figures 1.1, 1.2, 1.3). Unlike NHEJ, HR requires a sequence to be used as a template to repair the break.

When a DSB occurs, it is primarily detected by the direct interaction of the DNA ends with the MRN complex, composed of MRE11, RAD50 and NBS1, and this complex is involved in both NHEJ and HR (Maser et al., 1997). The MRN complex is required for the recruitment of the Ataxia Telangiectasia Mutated kinase (ATM), while the ATR-Interacting Protein (ATRIP) recruits the Ataxia Telangiectasia and Rad3-related protein (ATR) (Cortez et al., 2001; Falck et al., 2005; Uziel et al., 2003). ATM and ATR complexes phosphorylate different proteins, including the protein kinases CHK1 and CHK2, that together cooperate to induce cell cycle arrest (Gatei et al., 2003; Jazayeri et al., 2006; Liu et al., 2000; Matsuoka et al., 2000; Zhao and Piwnica-Worms, 2001). ATM also phosphorylates the histone variant H2AX (Rogakou et al., 1998). γ H2AX is recognized by the Mediator of DNA Damage Checkpoint protein 1 (MDC1), which is phosphorylated by Casein Kinase 2 (CK2). The phosphorylated MDC1 recruits the NBS1 subunit of the MRN complex and more ATM. In this way, there is an amplification of the signal, which causes a spread of the γ H2AX phosphorylation (Lou et al., 2006; Stucki et al., 2005). The spreading signal aids in chromatin remodeling which permits the further accumulation of MDC1, MRN and ATR and other proteins that are involved in the double strand break repair.

1.1.1. Non Homologous End Joining (NHEJ)

The Non Homologous End Joining pathway consists of four main steps: recognition of the damage, formation of a “bridge” that brings the two DNA ends together, a polymerase that performs the fill-in synthesis and a ligase that seals the extremities (Figure 1.1). In mammals, the MRE11/RAD50/NBS1, the DNA-dependent Protein Kinase (DNA-PK) and the DNA ligases IV are the core of NHEJ machinery (Baumann and West, 1998;

Ma et al., 2004). It is thought that MRN is the first complex that interacts with the duplex. The RAD50 subunit, which has a DNA binding domain, has a structural role in bridging the free DNA ends (Hopfner et al., 2002). KU heterodimer is then recruited at the early stage of NHEJ. KU consists two subunits, KU70 and KU80, which form a ring that encircles the duplex on both of the free ends and can translocate along it (Mimori et al., 1986; Walker et al., 2001). No enzymatic function of KU is known, but it is postulated that its primary function is to mark the DNA termini in order to attract specific polymerases, nucleases and ligases to the site of DNA damage. The Ku70/K80 heterodimer together with the DNA-dependent Protein Kinase Catalytic Subunit (DNA-PKcs) form a complex known as the DNA-PK holoenzyme. KU binds with great avidity the DNA ends and it recruits the DNA-PKcs to such sites, which is then activated (Gottlieb and Jackson, 1993; Suwa et al., 1994; West et al., 1998; Yaneva et al., 1997; Hammarsten and Chu, 1998). In particular, the N-terminal region of DNA-PKcs is required for it to localize at the site of the DSB and to bind to the KU/DNA complex, whereas the C-terminal region possesses the kinase activity (Davis et al., 2013). *In vitro* DNA-PKcs phosphorylates various substrates, such as Ku70/80, X-ray Repair Cross-Complementing protein 4 (XRCC4), XRCC4-Like Factor (XLF), Artemis, Werner helicase (WRN) and DNA ligase IV (Chan et al., 1999; Lee et al., 2004; Ma et al., 2005; Wang et al., 2004; Yannone et al., 2001; Yu et al., 2008). However the importance of these post-translational modifications *in vivo* have still to be demonstrated (Douglas et al., 2005; Wang et al., 2004; Yu et al., 2008; Yu et al., 2003). The favourite substrate for DNA-PKcs is DNA-PKcs itself. Auto-phosphorylation leads to a DNA-PKcs conformational change and its subsequent decoupling from the DNA ends, enabling the other NHEJ factors to reach the DNA ends (Hammel et al., 2010; Uematsu et al., 2007).

The next step of NHEJ repair consists of filling in the gaps between the strands and is carried out by DNA polymerases. Both DNA-PKcs and KU, together with the initial

bridging action of RAD50 subunits of MRN complex, appear to play an active role in tethering the two broken DNA termini together, maintaining the proximity of the DNA ends (Cary et al., 1997; DeFazio et al., 2002; Ogiwara and Kohno, 2011). If the double strand ends are not complementary, the DNA strands must be processed in order to have ligatable DNA ends. In vertebrates, the Artemis protein is the nuclease involved in most of the tailoring activity during NHEJ repair (Ma et al., 2004). Artemis has intrinsic 5' to 3' exonuclease activity but in the presence of DNA-PKcs can also acquire a 5' and 3' endonuclease activity. It has been suggested that Artemis is not activated through phosphorylation by DNA-PKcs but that DNA-PKcs autophosphorylation causes an alteration of the DNA conformation that facilitates the access of Artemis to the DNA overhang (Goodarzi et al., 2006; Ma et al., 2002).

In *S. Cerevisiae*, DNA Polymerase 4 (POL IV), a member of the Y-family of polymerases, has an important role in the fill-in synthesis at NHEJ junctions (Tseng and Tomkinson, 2002; Wilson and Lieber, 1999). Polymerase λ (POL λ) is the closest mammalian homologue, and with Polymerase μ (POL μ) are associated with KU and Ligase IV/XRCC4 complex and are involved in the gap filling in NHEJ (Fan and Wu, 2004; Ma et al., 2004; Mahajan et al., 2002; Nick McElhinny et al., 2005). It has been reported that POL μ and POL λ are able to add nucleotides in the presence of a discontinuous template, and POL μ , unlike POL λ , has the ability to replicate in a template-independent manner, allowing the ligation of incompatible DNA termini (Gu et al., 2007; Nick McElhinny et al., 2005).

Since DNA ligation requires a 5' phosphate and a 3' hydroxyl group, PolyNucleotide Kinase/Phosphatase (PNKP) is recruited at this step in order to catalyze the phosphorylation of 5'-OH termini and for the removal of any 3'-P groups. PNKP has also been found to interact with XRCC4 factor (Chappell et al., 2002; Jilani et al., 1999; Koch et al., 2004). At this point, DNA ligase IV/XRCC4 complex and XLF (also known as

Cernunnos) are recruited by KU at the site of damage (Nick McElhinny et al., 2000). XLF stimulates the DNA ligase IV/XRCC4 complex enzymatic activity, allowing the ligation of incompatible and mismatched DNA ends (Ahnesorg et al., 2006; Tsai et al., 2007).

NHEJ does not require an undamaged DNA molecule and it is able to rejoin what remains of the two DNA ends without using a homologous template. Although the DNA has been repaired, in most cases, this pathway results in the loss or addition of a few nucleotides at the broken DNA ends. Hence, NHEJ is considered error-prone and is typically associated with genomic alterations.

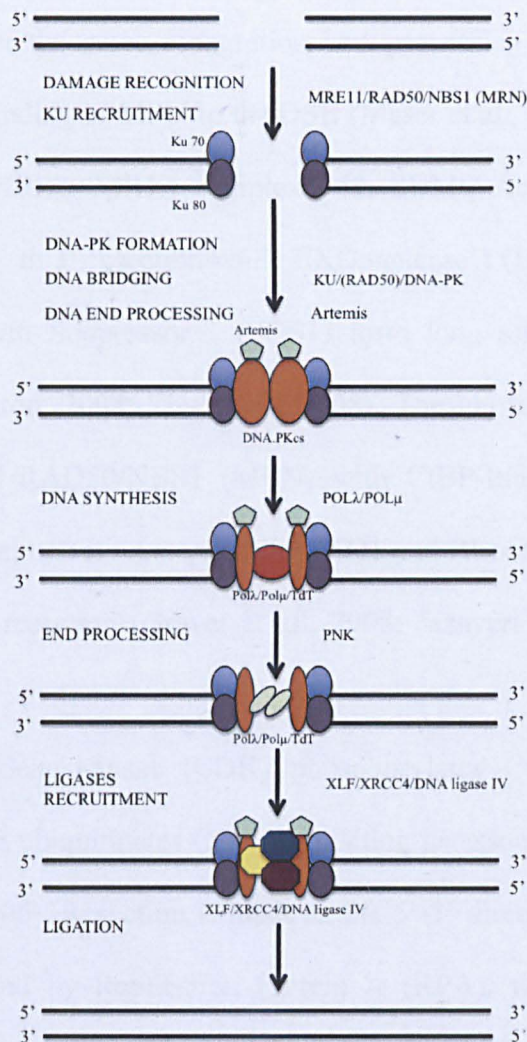


Figure 1.1. NHEJ pathway. In NHEJ, MRE11 recruits KU at the DSB DNA termini. KU recruits the DNA-PKcs and together with RAD50 bridge the DSB DNA termini. DNA-PKcs activates Artemis 5' to 3' endonuclease activity, which processes the DNA DSB ends. The DNA ends are then filled and re-ligated by the polymerases and ligases activity. Adapted from KEGG website (<http://www.genome.jp/kegg/pathway/ko/ko03450.html>).

1.1.2. Homologous Recombination (HR)

The fundamental differences between the NHEJ and the HR pathways are the requirement for a long stretch of homologous template DNA in HR (Figure 1.2). This homology increases the fidelity of the DSB repair. HR is mainly used during DNA replication in the S and G2 phase of the cell cycle, whereas NHEJ functions throughout the cell cycle and is the predominant mechanism for DSBs repair during G0, G1 and early S phase. Since HR involves copying of the missing information from an undamaged homologous chromosome it is considered an error free repair pathway without any loss of genetic information (Shrivastav et al., 2008).

HR starts with the same recognition and processing steps that are involved in NHEJ, which is the binding of MRN to the DSB (Maser et al., 1997) (Figure 1.2). In yeast, the MRE11/RAD50/XRS2 (MRX) complex with SUMO-Activating Enzyme subunit 2 (SAE2) initiates the 5' to 3' resection while EXOnuclease 1 (EXO1) and DNA2 nucleases with the Small Growth Suppressor 1 (SGS1) form long ssDNA (Gravel et al., 2008; Mimitou and Symington, 2008; Zhu et al., 2008). The human orthologous of the MRX complex, the MRE11/RAD50/NBS1 (MRN) with CtBP-Interacting Protein (CtIP) are important for the initial resection step, while EXO1 and BLoom syndrome protein (BLM) together perform the resection (Gravel et al., 2008; Jazayeri et al., 2006; Sartori et al., 2007).

Cyclin Dependent Kinase (CDK) phosphorylates CtIP that is recognized by BRCA1, which in turn ubiquitinates CtIP, stimulating its association with the site of DNA damage (Yu et al., 2006). Resection is made in the 5'-3' direction and results in a 3' end overhang that is bound by Replication Protein A (RPA), which serves to protect the ssDNA from further damage. To initiate the HR repair, RAD51 must be assembled on the single stranded filament. RAD55/RAD57 complex and RAD52 have been found to act as a recombinase mediators; in particular, they recruit RAD51, which displaces RPA from

ssDNA (Sung, 1997; Sugiyama and Kowalczykowski 2002). Also the BReast CAncer type 2 (BRCA2) has been found to act as a recombinase mediator by stimulating the binding of RAD51 to ssDNA in its ATP-bound form, preventing the binding of RAD51 to dsDNA (Carreira et al., 2009). The 3'-end-RAD51 nucleoprotein filament then "searches" on the sister chromatid for a homologous repair template and it then invades the homologous sequence forming a D-loop structure. RAD54 stabilizes the RAD51 nucleo-filament that in turn stimulates RAD54's ATPase activity, which is important for the chromatin remodelling during the strand invasion (Alexeev et al., 2003; Mazin et al., 2003; Wolner and Peterson, 2005).

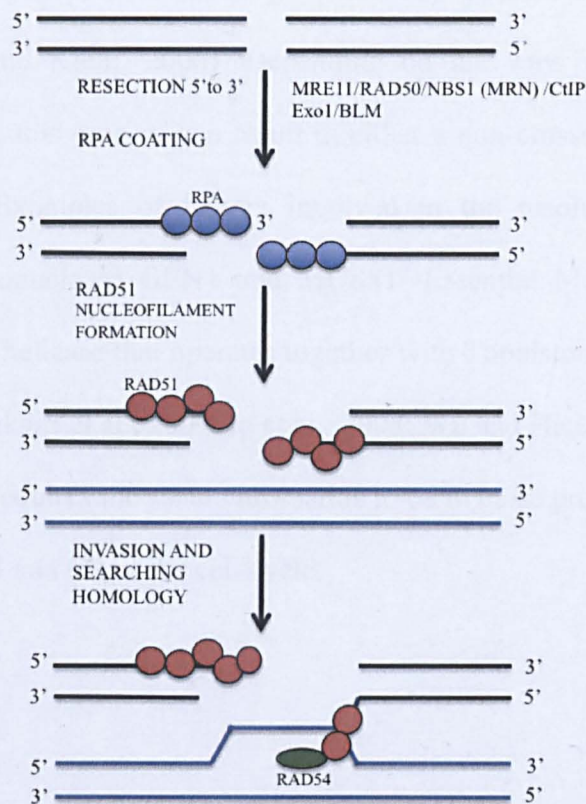


Figure 1.2. Initial steps of HR. In HR the MRN together with CtIP, EXO1, BLM resect the 5' ends at the DSB and the 3' ends are bound by the RPA proteins. RPA is then displaced and RAD51 is assembled on the 3' ends. RAD54 forms a complex with RAD51 and facilitates strand invasion and homology search. Adapted from (Sung and Klein, 2006).

In yeast, it has been reported that RAD54 stimulates heteroduplex extension and that both RAD54 and RAD51 ATPases activities are required for the disassembly of the

RAD51 nucleoprotein filament from the DNA overhang, in order to allow DNA synthesis machinery to reach the 3' end, whereas in higher eukaryotes, the RECQ helicases like BLM, perform this function (Bugreev et al., 2007; Li et al., 2007; Solinger and Heyer, 2001; Solinger et al., 2002).

At this point, two different HR processes known as Synthesis Dependent Strand Annealing (SDSA) and the Double Strand Break Repair (DSBR) sub-pathways can take place (Figure 1.3). In the SDSA process, the elongated 3' end is displaced out of the D-loop thus allowing it to re-anneal the DSB ends together through the newly synthesized complementary region. In DSBR the 3' overhang anneals to the other double strand when it is still part of the D-loop, thus creating a Holliday junction with the homologous chromosome (Sung and Klein, 2006). Depending on the way in which the Holliday junctions are resolved, this process can result in either a non-crossover or crossover gene conversion product. Examples of factors involved in the resolution of the Holliday junctions are the endonuclease GEN1 and MUS81/ Essential Meiotic Endonuclease 1 (EME1) and the BLM helicase that operates together with Topoisomerase III α (TOPOIII α) (Boddy et al., 2001; Fekairi et al., 2009; Ip et al., 2008; Wu and Hickson, 2003).

Therefore HR requires the sister chromatids to be in close proximity, which is more likely to occur in late S and G2 of the cell cycle.

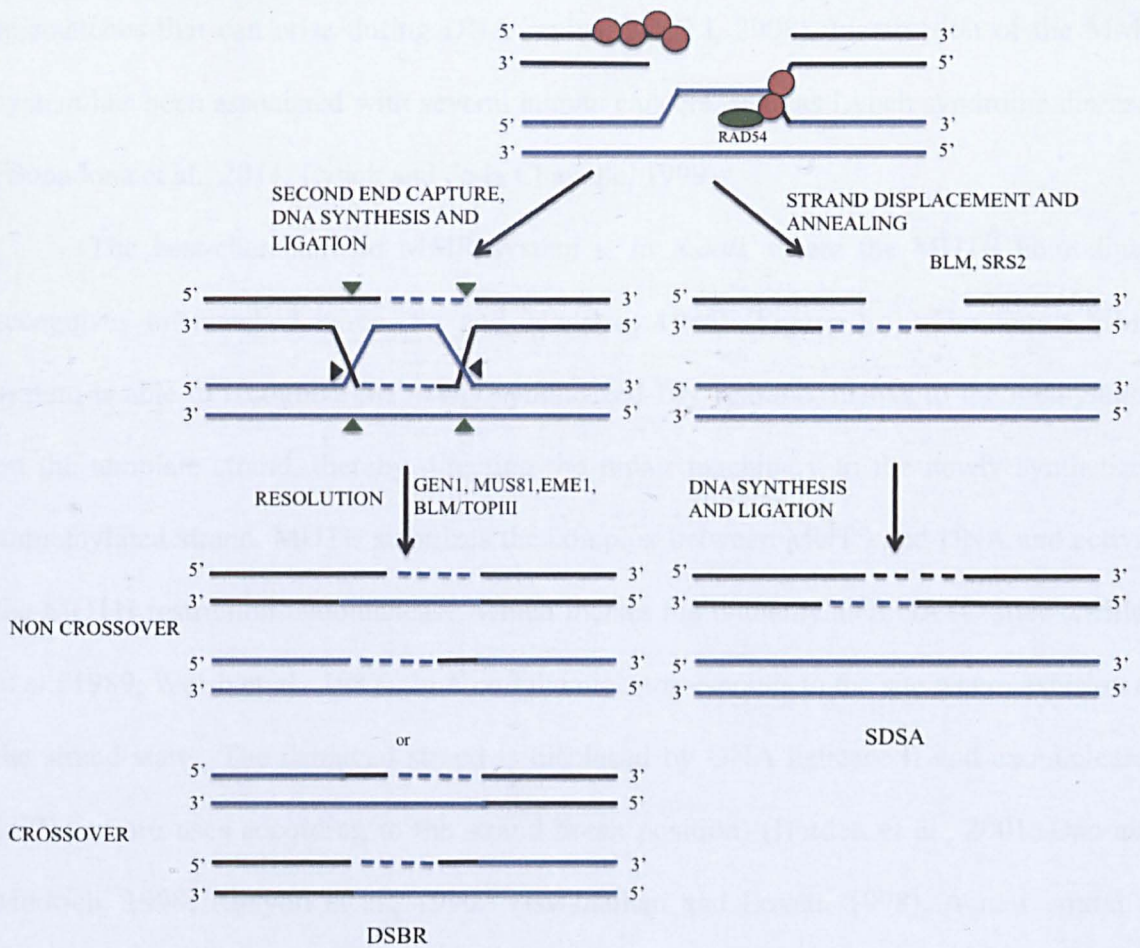


Figure 1.3. Final steps of HR. In HR the 3' overhang searches for the homology and invades the region in the duplex to form a (D)-loop structure. In the DSBR sub-pathway, after DNA synthesis, the second overhang also invades the other strand forming a Holliday junction with the homologous chromosome. Holliday junctions may be resolved by GEN1, MUS81, EME1, and BLM/TOPIII. Depending on the site of the cleavage, different product may be generated with or without crossover of the parental chromosomes. In the SDSA sub-pathway the elongated 3' overhang is displaced and re-annealed with the 5' end. Adapted from (Sung and Klein, 2006).

1.2. Single Strand Break Repair

1.2.1. Mismatch Repair (MMR)

Base mispairing that occurs during DNA replication and recombination can lead to genetic errors. Mismatch Repair pathway (MMR) is able to recognize and repair this mispairings, as well as short insertions and deletions; it is therefore an important contributor for genome maintenance. In fact, if DNA damage is not repaired, it can generate mutations leading to diseases. MMR prevents mutations by correcting the

mismatches that can arise during DNA replication (Li, 2008). Inactivation of the MMR system has been associated with several human cancers, such as Lynch syndrome diseases (Bonadona et al., 2011; Lynch and de la Chapelle, 1999).

The best-characterized MMR system is in *E.coli*, where the MUTS homodimer recognizes mismatched bases (Su and Modrich, 1986) (Figure 1.4). The *E.coli* MMR system is able to recognize the newly synthesized DNA strand, thanks to the methylation on the template strand, thereby directing the repair machinery to the newly synthesized unmethylated strand. MUTL stabilizes the complex between MUTS and DNA and activates the MUTH restriction endonuclease, which incises the unmethylated GATC sites (Grilley et al., 1989; Welsh et al., 1987). In *E.coli* the nick corresponds to the site where excision of the strand starts. The damaged strand is displaced by DNA helicase II and exonucleases (different are used according to the strand break position) (Burdett et al., 2001; Dao and Modrich, 1998; Runyon et al., 1990; Viswanathan and Lovett, 1998). A new strand is synthesized to fill the gap by DNA polymerase III. Finally the strands are closed by DNA ligase (Lahue et al., 1989).

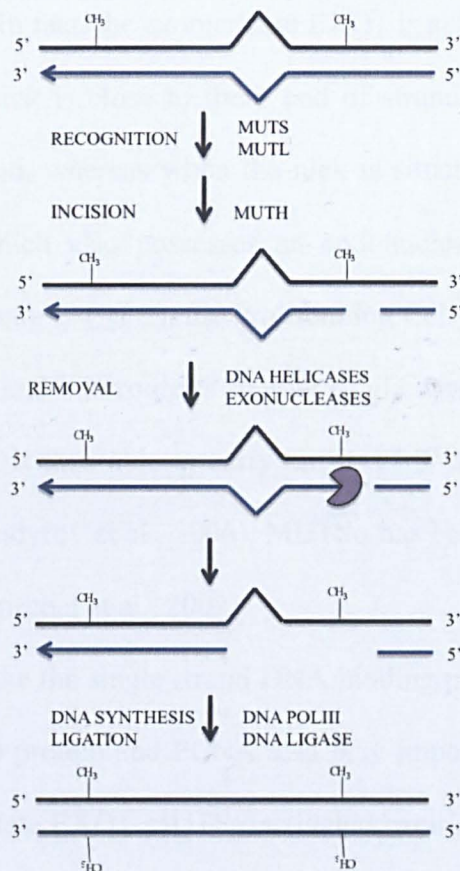


Figure 1.4. MMR in *E. coli*. Mismatch is recognized by MUTS and MUTL and then incised by MUTH, which is able to recognize the new unmethylated DNA strand (GATC site). The mismatch is then removed by four exonucleases and new DNA strand is re-synthesized by polymerase III. Adapted from KEGG website (http://www.genome.jp/kegg-bin/show_pathway?ko03430).

In eukaryotes, the site of the mismatch is recognized by two heterodimers: MUTS α , which is composed of MSH2 and MSH6 subunits, and MUTS β , which is composed of MSH2 and MSH3 subunits (Alani, 1996; Palombo et al., 1995) (Figure 1.5). MUTS α , is responsible for the recognition of base-base mismatches, including the insertion/deletion of one or two unpaired nucleotides, but is also capable of recognition of larger insertion/deletion with lower affinity. Instead, insertions/deletions from 2 to 8-10 mispairs are detected by MUTS β (Genschel et al., 1998).

For subsequent steps, the MUTL α heterodimer (MLH1/PMS2) is recruited and the strand that contains the mismatch is excised. In mammals, two different excision systems have been proposed to take place depending on where the nick in the newly synthesized

DNA strand is situated. In fact, the exonuclease EXO1 is able to degrade DNA only in a 5' to 3' direction; if the nick is close to the 5' end of strand, the exonuclease activity can proceed toward the 3' end, whereas when the nick is situated close the 3' end, the PMS2 subunit of MUTL α , which also possesses an endonuclease activity, is activated in a MUTS α , Replication Factor C (RFC), the Proliferating Cell Nuclear Antigen (PCNA), and ATP-dependent manner and it introduces another single strand break in the strand opposite of the mismatch. EXO1 is then able to carry out its 5'-3' activity (Genschel et al., 2002; Dzantiev et al., 2004; Kadyrov et al., 2006). MUTS α has been reported to activate the 5' to 3' activity of EXO1 (Genschel et al., 2002).

Other proteins, like the single strand DNA binding protein RPA, the High Mobility Group Box 1 (HMGB1) protein and PCNA also play important roles in MMR. RPA and HMGB1 together stimulate EXO1 MUTS α -activated excision, but RPA has a role also in the MUTL α -dependent suppressing EXO1-excision activity upon mismatch removal (Zhang et al., 2005). PCNA has a role in the early steps of MMR (Umar et al., 1996). In fact it has been found to interact with MUTS α and MUTS β , but also weakly with MUTL α via their PCNA binding motif (Clark et al., 2000; Kleczkowska et al., 2001; Lee and Alani, 2006). PCNA is also thought to play a role in the later steps of MMR acting as a processivity factor during DNA re-synthesis (Gu et al., 1998).

Finally, the gap is filled by the DNA polymerase δ and then DNA ligase I closes the nicks between the strands (Zhang et al., 2005).

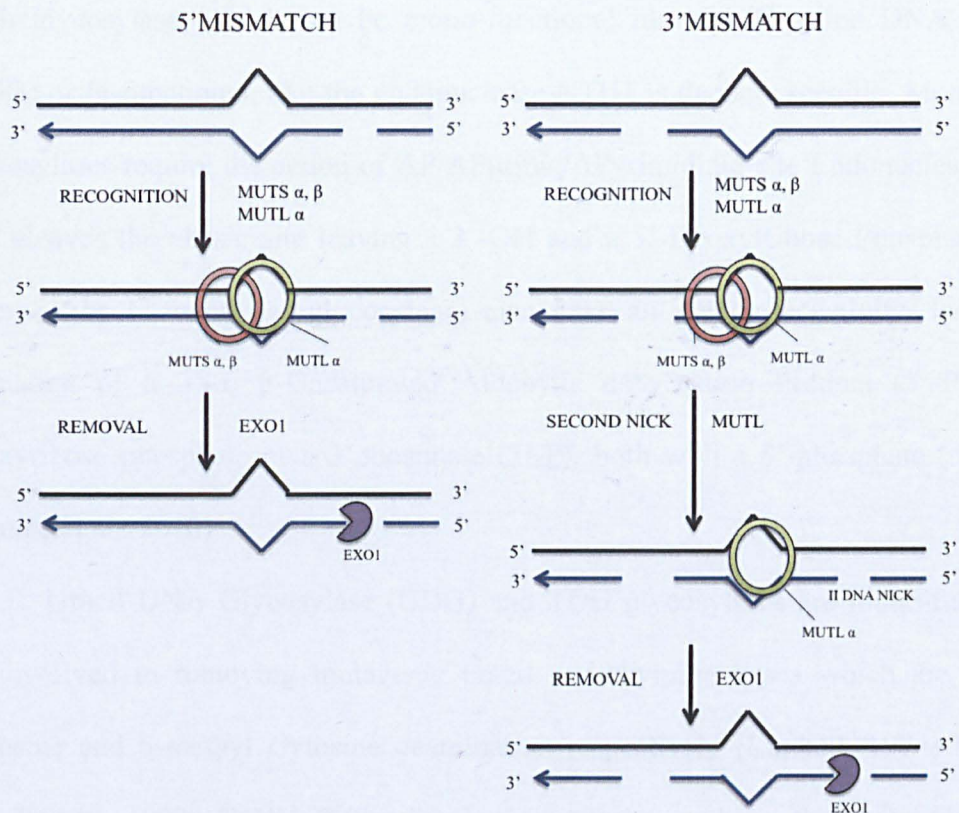


Figure 1.5. Two different initial steps in Eukaryotes MMR. When the nick is located close to the 5' termini, 5'-3' EXO1 exonuclease activity takes place. If the nick is situated close to the 3' termini, another DNA nick is produced by MUTLα. At this point, EXO1 is able to perform its activity. Adapted from KEGG website (http://www.genome.jp/kegg-bin/show_pathway?ko03430).

1.2.2. Base Excision Repair (BER)

DNA hydrolysis can cause the formation of abasic sites and deamination reactions can lead to the conversion of one base with another, such as a cytosine to uracil. ROS that arise from aerobic metabolism leads to many types of modification, such as the formation of 8-hydroxyguanine. BER pathway is involved in the repair of most of these endogenous DNA lesions and it is characterized by five steps (Figures 1.6, 1.7). These are the recognition of the base and its excision, the removal of the abasic site, the modification of the DNA break ends, the filling of the gap and the ligation of the two DNA ends (Nemec et al., 2010).

BER starts with the excision of a damaged base by a DNA glycosylase (Figure 1.6).

Each glycosylase, which can be mono-functional like the Thymine DNA Glycosylase (TDG) or bi-functional, like the endonuclease NTH1 is damage specific. Mono-functional glycosylases require the action of AP APurinic/APyrimidinic site Endonuclease 1 (APE1) that cleaves the abasic site leaving a 3'-OH and a 5'-DeoxyRibose Phosphate (5'-dRP), whereas the bi-functional glycosylases also have an AP lyase activity, leading to the formation of a 3'- α , β -Unsaturated Aldehyde dehydration Product (3'-PUA) of the deoxyribose phosphate or a 3'-phosphate (3'-P), both with a 5'-phosphate (5'-P) termini (Nemec et al., 2010).

Uracil DNA Glycosylase (UDG) and TDG glycosylases are mono-functional and are involved in removing mutagenic uracil and thymine bases which are products of Cytosine and 5-methyl Cytosine deamination respectively (Lindahl, 1974; Neddermann and Jiricny, 1993, 1994). Bifunctional glycosylases, such as the 8-OxoGuanine-DNA Glycosilase (OGG1), the NEILs family and NTH1 are specific for oxidized bases, such as 8-oxo-guanine (Bandaru et al., 2002; Hazra et al., 2002; Matsumoto et al., 2001; van der Kemp et al., 1996).

The two ends of the break must be modified in order to produce a 3'-OH and 5'-P break end. In particular, the 5'-dRP processing is carried on by polymerase β (Matsumoto and Kim, 1995; Prasad et al., 1998), while the 3'-PUA and the 3'-P are modified by APE1 and PolyNucleotide Kinase/Phosphatase (PNKP) respectively (Das et al., 2006; Suh et al., 1997; Wiederhold et al., 2004).

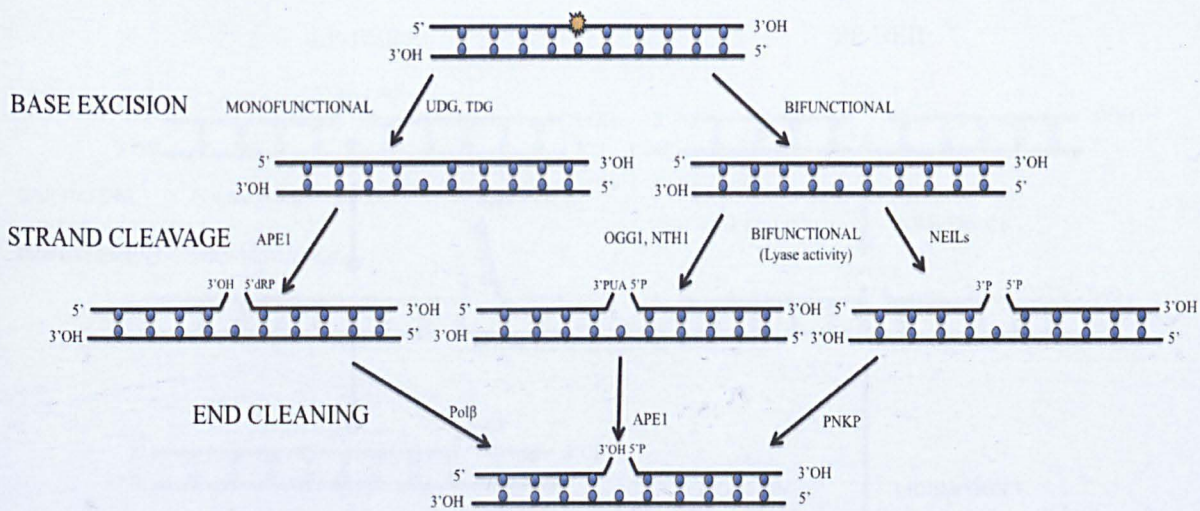


Figure 1.6. Initial steps of BER. BER starts with the removal of the damaged base by a DNA glycosylase. Monofunctional glycosylases like UDG and TDG require the action of APE1 endonuclease that cleaves the abasic site, leaving a 3'-OH and a 5'-dRP ends. 5'-dRP end is then modified by POL β , in order to produce a 5'-P end. Bifunctional glycosylases, such as OGG1 and NEILs possess also the abasic site lyase activity, leaving a 5'-P and a 3'-PUA or 3'-P-ends. 3'-PUA and 3'-P are modified by the action of APE1 and PNKP. Adapted from (Hegde et al., 2008).

At this level, two different sub-pathways of BER, named Short Patch (SP) and Long Patch (LP) repair may be activated according to the type of lesion (Frosina et al., 1996) (Figure 1.7). In the SP repair, a single nucleotide is removed and then repaired through a reaction catalysed mainly by the DNA polymerase β , which adds one nucleotide to the 3' end of the nick (Sobol et al., 1996). At the last step, XRCC1 and the ligase Ligase III α are required for the sealing of the nick (Cappelli et al., 1997). The LP BER, which results in the replacement of approximately 2-10 nucleotides including the damaged base, occurs when polymerase β is not able to remove the 5'-dRP lesion. The filling of the gap requires the DNA polymerases δ , ϵ and β (Harrigan et al., 2003; Prasad et al., 2001; Stucki et al., 1998). WRN, together with Poly (ADP-Ribose) Polymerase 1 (PARP1) and Flap structure-specific EndoNuclease 1 (FEN1) stimulate DNA polymerase β 5'-dRP strand displacement and flap DNA substrate is excised by the FEN1 flap nuclease activity and at a final step, DNA Ligase I (LIG I) ligates the DNA termini (Prasad et al., 2000; Harrigan et al., 2003; Klungland and Lindahl, 1997; Prasad et al., 2001).

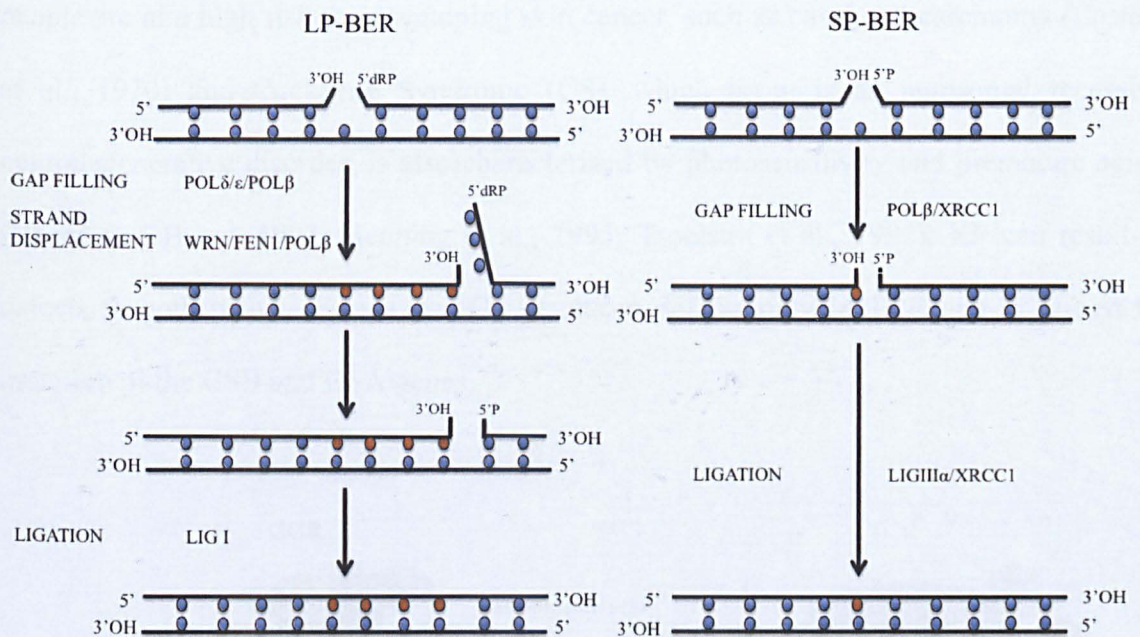


Figure 1.7. Final steps of BER. Long Patch (LP) repair and Short Patch (SP) repair are represented. The LP repair occurs when POL β does not remove 5'-dRP lesions. At this point, POL δ , ϵ and β together fill the gap between the two DNA termini, displacing the 5'-dRP of 2-10 nucleotides. FEN1 together with WRN promote the strand displacement and excision. In the SP repair, a single nucleotide is removed and POL β adds a nucleotide at the 3'-OH end. The final gaps for SP and LP are the same and consist of the ligation of the DNA termini, by LIGI and LIGIII α /XRCC1, respectively. Adapted from (Hegde et al., 2008).

1.2.3. Nucleotide Excision Repair (NER)

The NER system is able to repair different types of damage; in fact it recognizes the distortion caused by the lesion in the DNA double helix (Figure 1.8). NER is related to BER, but the NER system is used when the modification of the bases is larger (2-30 nucleotides) and/or causes a distortion in the double helix due to, for example, the formation of thymine dimers. Two sub-pathways characterize NER, the Global Genome Repair (GGR), which repairs the lesions over the entire genome and the Transcription-Coupled Repair (TCR), which has the role of repairing the lesions that block the transcription (Figure 1.8). GGR and TCR are similar except for the initial recognition step of the DNA damage.

The NER pathway involves several disorders, including Xeroderma Pigmentosum (XP) (from XPA to XPG type), which is characterized by increased UV sensitivity and

people are at a high risk for developing skin cancer, such as basal cell carcinoma (Epstein et al., 1970) and Cockayne Syndrome (CS), which is an autosomal recessive neurodegenerative disorder, is also characterized by photosensitivity and premature aging (Nance and Berry, 1992; Henning et al., 1995; Troelstra et al., 1992). XP can result in defects to both pathways, whereas CS results in defects only in TCR and is caused by mutation of the CSB and CSA genes.

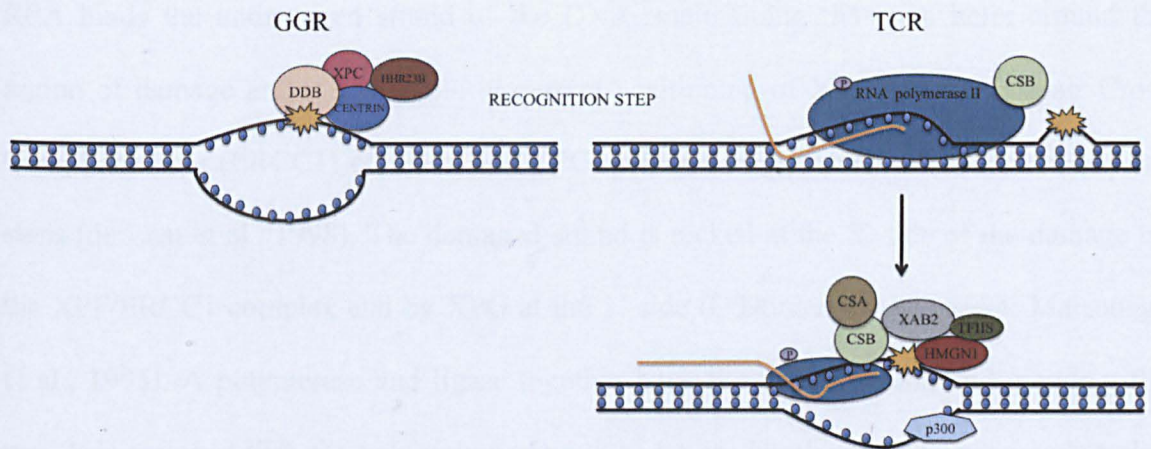


Figure 1.8. Initial steps of NER. Here two two recognition steps are represented. In GGR sub-pathway, the helix distorting lesion is recognized by a complex formed by XPC, HHR23B and Centrin2 and by DDB complex. In TCR the RNA POLII is blocked when encounters a DNA damage and CSB is recruited at the damaged site. Subsequently, p300, CSA, XAB2, HMGNI and TFIIS factors are recruited to remodel the chromatin. Adapted from (Kamileri et al., 2012).

In GGR, the complex formed by XPC, Human Rad23 Homologue B (HHR23B) and Centrin 2 has the task of recognizing the helix distorting lesion (Araki et al., 2001; Batty et al., 2000; Sugasawa et al., 1998; Sugasawa et al., 2001) (Figure 1.8). Since the XPC complex is not able to recognize small structural perturbations to the DNA, such as Cyclobutane Pyrimidine Dimers (CPDs), another complex called the DNA Damage Binding (DDB) complex, is involved in this reaction (Fitch et al., 2003). This complex is formed by two subunits, known as DDB1 and DDB2/XPE. The latter has been found to have a high affinity for several DNA lesions, CPD dimers in particular (Wittschieben et al., 2005).

At the pre-incision step, another factor, known as Transcription Factor II H (TFIIH), is recruited by XPC (Yokoi et al., 2000) (Figure 1.9). The TFIIH core contains the subunits XPB, XPD, p62, p52, p44, p34, and p8/TTD-A, which are coupled to a Cdk-Activating Kinase complex (CAK). The two helicases XPB and XPD of the TFIIH complex unwind the DNA at the site of damage (Coin et al., 2007). CAK, which has been found to inhibit the XPD DNA unwinding activity, is released from the TFIIH complex by the XPA enzymatic activity (Coin et al., 2008; Sandrock and Egly, 2001). At this point, RPA binds the undamaged strand of the DNA, maintaining the open helix around the region of damage and it has a role in correct positioning of XPF/Excision Repair Cross Complementing (ERCC1) complex and XPG endonucleases, which act in the subsequent steps (de Laat et al., 1998). The damaged strand is nicked at the 5' side of the damage by the XPF/ERCC1 complex and by XPG at the 3' side (O'Donovan et al., 1994; Matsunaga et al., 1995). A polymerase and ligase together have the task of filling and mending the gap. It is assumed that not only polymerases δ and ϵ are involved in this process but also the translesion polymerase κ (Ogi et al., 2010).

The repair steps of TCR-NER are similar to those of GGR-NER, but in case of TCR, the signal for the repair is the blockage of transcription elongation by RNA Polymerase II (RNAPII) upon lesion formation (Figure 1.8). It has been demonstrated that CSB is the key factor in TC-NER. A stable RNAPII/CSB complex is assembled and then the other pre-incision and incision factors, which are shared with the GGR pathway, bind to this complex (Fousteri et al., 2006; Tantin et al., 1997). CSB is also required for the recruitment of the histone deacetylase P300 to the stalled polymerase and the CSA. CSA recruits additional TC-NER specific factors such as the High Mobility Group Nucleosome 1 (HMGN1), which is a chromatin remodeling factor, the transcription elongation factor TFIIS and the pre-mRNA splicing factor XPA Binding protein 2 (XAB2) (Fousteri et al., 2006). HMGN1, together with p300, play a role in chromatin remodeling, facilitating

access to the DNA (Birger et al., 2003; Fousteri et al., 2006). One other function of CSB may be to remove RNAPII from the damage site, so the repair can take place (Tijsterman and Brouwer, 1999).

When the chromatin is in a more open form, the NER core is able to carry out the repair (Figure 1.9).

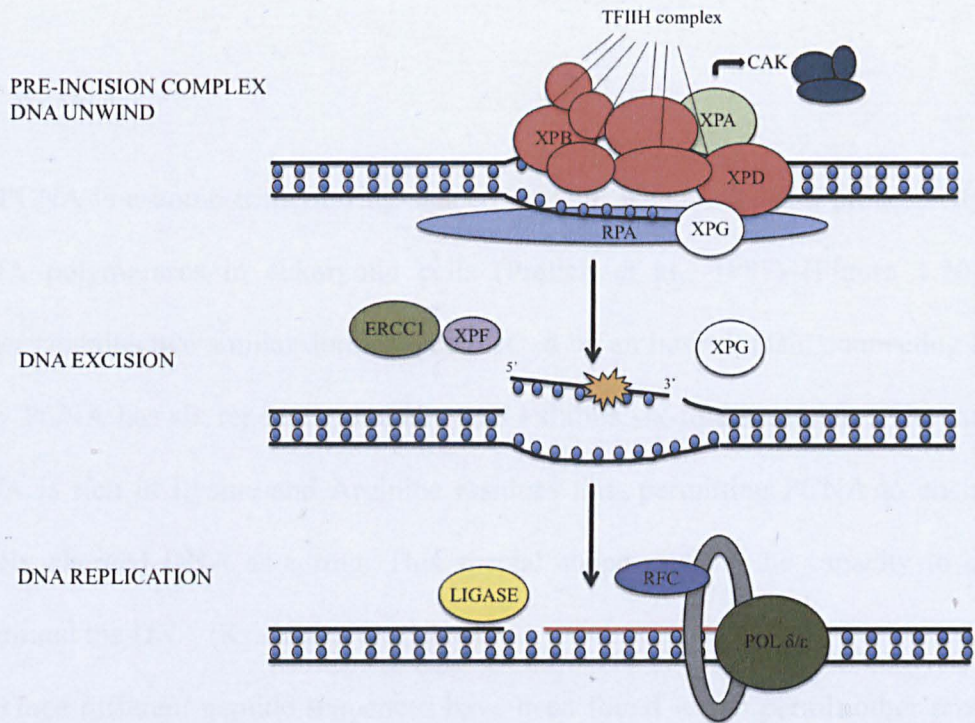


Figure 1.9. Final steps of NER. At the pre-incision step TFIIH complex is recruited. XPB and XPD subunits of TFIIH complex unwind the DNA at the site of the damage. RPA binds the undamaged strand and positions XPG and XPF/ERCC1 endonucleases that nick the damaged strand. Polymerase δ, ϵ and κ are involved in the gap filling. Adapted from (Kamileri et al., 2012).

1.3. Damage Tolerance

During S phase of the cell cycle, the promotion of the completion of DNA replication rather than protecting the accuracy of the genomic information is preferred. Therefore, cells have evolved schemes to ensure continuation of DNA synthesis in the presence of damage, using a mechanism known as damage tolerance. In this case it is possible to bypass the lesion. Since this pathway does not remove the lesion from the

DNA, it results in mutations.

Two pathways of DNA damage tolerance have been described, known as the error prone DNA TransLesion Synthesis (TLS) and the Error Free lesion bypass (Figure 1.11, 1.12). Recent studies have described how the ubiquitination of PCNA plays an important role in coordinating DNA replication and the DNA damage tolerance process (Strzalka and Ziemienowicz, 2011) (Figure 1.10).

1.3.1. PCNA

PCNA is a homo-trimeric ring-shaped protein, which acts as a processivity factor for DNA polymerases in eukaryotic cells (Prelich et al., 1987) (Figure 1.10). Each monomer contains two similar domains, connected by an inter-domain connecting loop; in this way PCNA has six repeating domains and exhibits six-fold symmetry. The inner part of PCNA is rich in Lysine and Arginine residues thus permitting PCNA to encircle the negatively charged DNA as a ring. This special shape gives it the capacity to act as a clamp around the DNA (Krishna et al., 1994).

Three different peptide sequences have been found which permit other proteins to interact with PCNA: the PCNA Interacting Peptide (PIP) is contained in DNA polymerase η , κ and P21 (Haracska et al., 2001; Haracska et al., 2002; Warbrick et al., 1995), the KA box in polymerase ϵ , δ and RFC (Xu et al., 2001) and the ALKB homologue 2 PCNA-Interacting Motif (APIM), has been characterized in Human AlkB Homologue-2 (hABH2) protein (Gilljam et al., 2009). Therefore, PCNA acts as a scaffold to tether proteins to the DNA. When PCNA tethers a polymerase to the DNA, it acts as a processivity factor by keeping the polymerase associated with the DNA.

At the beginning of DNA replication, polymerase- α /primase synthesizes the first RNA/DNA primer on the strand. The chaperone-like RFC complex recognizes the RNA primer terminus along the DNA and loads PCNA onto the double stranded region (Podust

et al., 1995). Polymerases together with PCNA perform the DNA synthesis. In particular, the elongation of the leading strand is mediated by polymerase α and polymerase ϵ , whereas polymerase α and δ conduct the elongation of the lagging strand (Garg and Burgers, 2005).

If the replication machinery encounters DNA damage, DNA replicative polymerases do not have the capacity to bypass the lesion and the replication fork stalls. It has been shown that after DNA damage, PCNA can be either mono-ubiquitinated or poly-ubiquitinated (Hoege et al., 2002) (Figure 1.10). The ubiquitination and de-ubiquitination process will be discussed later.

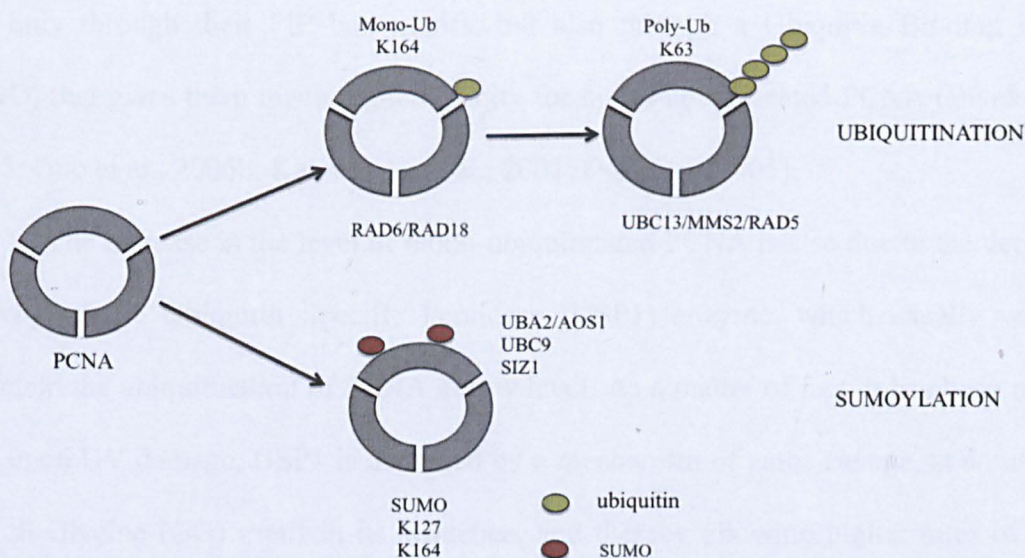


Figure 1.10. PCNA ubiquitination and sumoylation. PCNA can be modified by either ubiquitin at K164 or SUMO at K164 and K127. Both modifications take place during S phase of cell cycle. Mono-ubiquitination recruits TLS polymerases, whereas poly-ubiquitinated PCNA is recognized by an unknown factor, leading to an error free pathway. PCNA sumoylation appears to recruit anti-recombinase proteins in order to avoid unnecessary homologous recombination. Adapted from (Hoege et al., 2002).

1.3.1.1. Translesion Synthesis

Two different models for the TLS bypass have been proposed; the first, known as translesion synthesis, consisted of a polymerase-switch (Figure 1.11), where the mono-ubiquitination of PCNA is the key step and the second, called gap-filling models, involves another processivity factor.

Translesion synthesis is a highly conserved process, which allows the cells to bypass DNA damage using low fidelity DNA polymerases (Figure 1.11). It is characterized by two steps, involving the RAD18 E3 ligase and the RAD6 E2, which interact forming a complex and consists in the linkage of a single ubiquitin to K164 of PCNA (Hoege et al., 2002). In the polymerase switch model, mono-ubiquitination of PCNA causes a “polymerase switch” in which TLS polymerases like polymerase η and κ and Rev1, all of which belong to the Y-family of DNA polymerase, and polymerase ξ , a B-family polymerase, replace the more processive B-family polymerases, such as α , δ and ϵ (Kannouche et al., 2004). This happens because the TLS polymerases interact with PCNA not only through their PIP box motifs, but also through a Ubiquitin Binding Domain (UBD) that gives them much higher affinity for mono-ubiquitinated PCNA (Bienko et al., 2005; Guo et al., 2006b; Kannouche et al., 2001; Ogi et al., 2005).

The increase in the level of mono-ubiquitinated PCNA is also due to the decreasing activity of the Ubiquitin Specific Peptidase (USP1) enzyme, which usually works to maintain the ubiquitination of PCNA at low level. As a matter of fact, it has been reported that upon UV damage, USP1 is degraded by a mechanism of autocleavage, at an ubiquitin like di-Glycine (GG) motif in its sequence, and thereby allowing higher rates of PCNA ubiquitination (Huang et al., 2006).

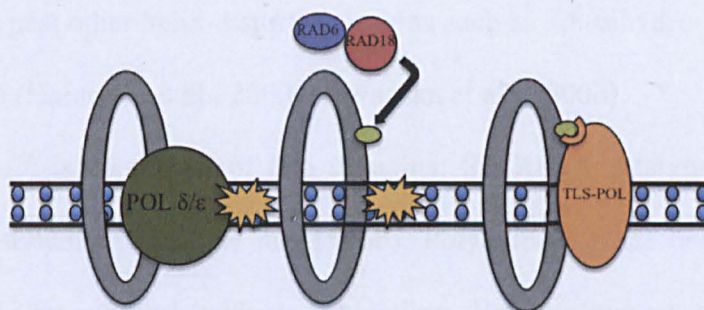


Figure 1.11. Polymerases switching. Replication machinery stalls at the site of DNA damage. RAD6/RAD18 ligases ubiquitinate the PCNA at the K164. The increasing affinity of the UBZ containing TLS polymerases for the mono-ubiquitinated PCNA leads to a polymerase switch. TLS replicate past the lesion. Adapted from (Hofmann, 2009).

The distinguishing feature of the Y-family of DNA polymerases is the ability to replicate through DNA lesions. As a matter of fact they can accommodate bulky adducts, like thymine dimers in their active sites. TLS polymerases have a lower processivity than the replicative polymerases and also lack the 3'-5' proofreading activity, since the appropriate domain is not present (Waters et al., 2009).

The TLS polymerases are able to bypass different type of lesion, as shown in Table 1.2. For example, polymerase η is specialized in the replication of the CPDs, which is one of the major types of damage caused by UV irradiation (Masutani et al., 1999). Polymerase η has an active site, which is large and that allows it to accommodate the entire dimer; polymerase η replicates the CPDs with the same efficiency as undamaged DNA (Washington et al., 2000; Yoon et al., 2009). Polymerase η appears to be the less mutagenic polymerase in bypassing the UV-induced DNA lesion (Johnson et al., 1999). In cells from Xeroderma Pigmentosum V-type (XPV) patients, polymerase η is not expressed, leading to a high UV sensitivity and to a predisposition of the patient to develop skin cancer (Masutani et al., 1999). This effect is caused by polymerase η being replaced by three polymerases, which are less accurate and thus more mutagenic: the model describes that polymerase κ and ι perform the insertion step and collaborate with the polymerase ξ , which performs the extension step (Ziv et al., 2009). Polymerase η has the ability to replicate past other helix-distorting lesions such as 7,8-dihydro-8-oxoguanine and O6-methylguanine (Haracska et al., 2000a; Haracska et al., 2000b).

Polymerase ξ is composed of two domains: the REV3 catalytic subunit and the REV7 accessory subunit (Nelson et al., 1996b). Polymerase ξ has been reported to be involved in TLS for several adducts, including Benzo(a)pyrene adducts (BP-Gs), Cisplatin-GG adducts (cisPt-GGs) as well as across Thymine-Thymine Pyrimidine-Pyrimidone (6-4) (TT 6-4 PP) (Shachar et al., 2009). Polymerase ξ is inefficient in

inserting a nucleotides opposite the lesion, but, as previously mentioned, has a more general role in performing the extension step in two-polymerase lesion bypass (Johnson et al., 2000). As an example, it has been reported that BP-Gs are bypassed in reactions that involves three different TLS polymerases; in particular, the insertion of the base appears to be carried out by polymerase η and κ , while the new strand of DNA is extended by polymerase ξ , resulting in a error free or error-prone TLS according to the insertion polymerase that is involved (Shachar et al., 2009).

Polymerase ι has a peculiar characteristic; it has different accuracy according to the template base. When the template is an adenine, it is changed to a *syn* conformation by the two residues of the polymerase finger domain Q59 and K60, resulting in Hoogsteen base pairing, in which only the thymine in an *anti* conformation can be matched. On the other hand, when the template is the thymine, it is maintained in the *anti* conformation and the incorporation of an *anti* guanine, which has a largest stacking surface, is preferred over an incoming adenine in *syn* conformation (Nair et al., 2004). It also allows the correct replication of the 8-oxoguanine adduct that can be correctly matched with an incoming cysteine base (Kirouac and Ling, 2011).

REV1 exclusively inserts the dC nucleotide opposite the template dG, bulky guanines and abasic sites, for this reason, REV1 is known as a dCMP transferase (Nelson et al., 1996a). REV1 has a particular mechanism to select the incoming dC nucleotide. The R324 residue of REV1 forms hydrogen bound with the nucleotide avoiding the usual direct interaction between the bases pairing (Nair et al., 2005). REV1 possesses a BRCT domain, which binds the REV7 subunit of polymerase ξ . The BRCT domain, together with an Ubiquitin Binding Motif (UBM), mediates the interaction between REV1 and PCNA and are important for the REV1 nuclear foci localization (D'Souza and Walker, 2006; Guo et al., 2006a; Guo et al., 2006b). In this way, besides its role in transferase activity, REV1 interacts with the other polymerases, suggesting that it has an important role as TLS

polymerase scaffold (Guo et al., 2003; Ohashi et al., 2004; Tissier et al., 2004).

Once TLS polymerases replicate past the lesion, PCNA is de-ubiquitinated by DUB enzymes, like USP1, and TLS polymerases are replaced by the more accurate replication polymerases (Huang et al., 2006).

GENE	PROTEIN	ROLE (examples)
REV1	REV1	Incorporation dC opposite dG Scaffold for other TLS proteins
POL H (RAD30A/XPV)	POL η	Replication past CPD
POLI (RAD30B)	POL ι	Incorporation dT opposite dA
POLK (DINB1)	POL κ	Replication past BP-G, cisPt, NER
REV3L	POL ζ	Extension step

Table 1.2. TLS polymerases. Y-family polymerases (REV1, POL η , POL κ , POL ι) and the B-family POL ξ are involved in the translesion synthesis.

In the gap-filling model, the TLS is performed to fill the gaps that are left by the replication machinery, like the ones that arise from the re-priming of the leading strand or the initiation of a new Okazaki fragment (Heller and Marians, 2006). As it has been reported in yeast, it is mainly employed during the G2/M phase of the cell cycle. It seems that the TLS polymerases that are involved in the polymerase switch TLS, are also involved in the gap-filling model, since they are still expressed outside S-phase (Karras and Jentsch, 2010).

1.3.1.2. Error Free Pathway

PCNA can also be poly-ubiquitinated by the ubiquitin ligase RAD5 in cooperation with an E2 enzyme composed of UBC13 and MMS2 subunits, leading to the formation of a K63-linked poly-ubiquitin chain (Brusky et al., 2000; Hofmann and Pickart, 1999; Ulrich

and Jentsch, 2000) (Figure 1.12). Little is known about the resulting downstream process; an unknown factor recognizes the poly-ubiquitinated PCNA and initiates the so-called error-free pathway.

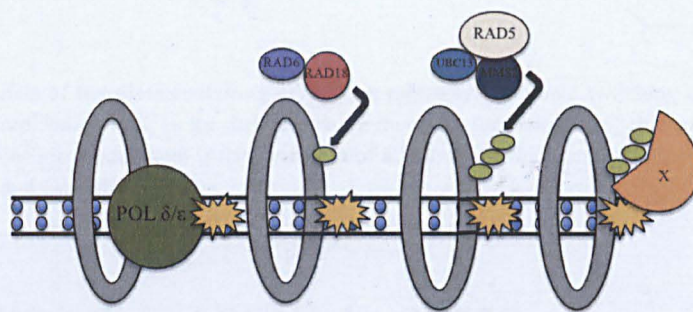


Figure 1.12. Error free pathway. PCNA can be poly-ubiquitinated by the complex formed by RAD5/MMS2/UBC13 ligases. Poly-ubiquitin chain is then recognized by an unknown factor (orange X), leading to an error free pathway. Adapted from (Hofmann, 2009).

Two possible models for error free-lesion bypass (named also template switching) have been proposed, namely recombination and replication fork regression (Figure 1.13). In the fork regression model, the nascent single strand encounters damage in the parental strand and there is a regression of the replication fork, which is unwound to generate a four-stranded DNA structure called a “chicken foot”. In this way the new filament of the strand is paired with the nascent filament of the other strand, which is used as a template to continue the replication, and the DNA lesion is relocated back into the reformed parental duplex. Eventually, fork progression is restored (Chang and Cimprich, 2009; Postow et al., 2001). In the recombination mediated template model there is the formation of a D-loop and the nascent strand that encounters the damage invades the other strand (Minca and Kowalski, 2010; Chang and Cimprich, 2009).

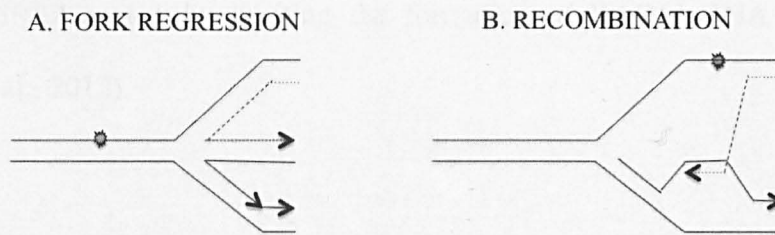


Figure 1.13. Two models of template switching error free pathway. Template switching consists of a rearrangement of the structure of the replication fork. In the fork regression there is a formation of a “chicken foot” structure (left side), whereas in the recombination model there is the formation of a D-loop in which one strand invades the other (right side). Adapted from (Chang and Cimprich, 2009).

1.3.1.3. Modification of PCNA by SUMO

In addition to ubiquitination, PCNA can also be modified by sumoylation (Figure 1.10). Like ubiquitination, sumoylation takes place in the S-phase of the cell cycle (Hoege et al., 2002) and both modifications of PCNA occur at K164. However it has been demonstrated in *S. cerevisiae* that when K164 is mutated, sumoylation can also occur at K127 (Hoege et al., 2002).

The consequence of sumoylation of PCNA is still unclear. In yeast, PCNA sumoylation may result in the recruitment of the helicase enzyme SRS2 that disrupts the RAD51 nucleoprotein filaments preventing unwanted recombination events during S-phase (Papouli et al., 2005; Pfander et al., 2005). In particular, SRS2 has been suggested to prevent HR by stimulating an ATP-hydrolysis within RAD51 filament, thus driving RAD51 to be liberated from the DNA (Antony et al., 2009). It has been demonstrated that the binding of SUMO to PCNA can prevent the interaction between PCNA interacting proteins and PCNA. As a matter of fact, sumoylation of PCNA represses the Establishment of COhesion protein 1 (ECO1)-dependent cohesion in yeast (Moldovan et al., 2006).

Recently, sumoylation of PCNA has been characterized in humans where it has been reported to restrict DSB formation and unnecessary recombination when the replication fork stalls (Gali et al., 2012). The human PCNA Associated Recombination Inhibitor (PARI) shows a high affinity for the SUMO/PCNA and has been demonstrated to

work as an SRS2 analog by limiting the formation of RAD51-DNA nucleofilaments (Moldovan et al., 2012).

SECTION B: Ubiquitination

Ubiquitin is a highly conserved protein found from yeast to mammals. Ubiquitination is a post-translational modification process that results in the covalent bond forming between ubiquitin and another protein; specifically it involves the carboxylic acid of the terminal Glycine of the ubiquitin and the amine of a Lysine residue on the target protein. It mainly serves as a signal for proteolytic degradation and as a scaffolding molecule in the formation of protein-protein interaction.

1.1. Ubiquitin

Ubiquitin is a 76 amino-acid polypeptide that possesses 7 Lysine residues and has a molecular mass of 8.5 kDa. It is present in all eukaryotic cells and is found throughout the cell. Ubiquitin is encoded by four different genes, namely UBA52, UBA80, UBB, UBC and all of them express ubiquitin as a large precursor (Ozkaynak, Finley et al. 1984; Lund et al., 1985). In particular the UBA gene codes for a single copy of ubiquitin fused to a ribosomal proteins L40 and S27 and the UBB and UBC genes code for a polyubiquitin head to tail fused precursor proteins (Wiborg et al., 1985). In order to have a mature free ubiquitin, the fusion protein must be processed. De-UBiquitinating (DUBs) enzymes cleave the peptide bonds between the ubiquitin molecules obtaining a 76 amino acids containing protein (Finley et al., 1989; Lund et al., 1985; Ozkaynak et al., 1987; Ozkaynak et al., 1984).

1.1.1. Ubiquitination Enzymes

The ubiquitination pathway initiates with the activation of ubiquitin (Ub) by the Ub-activating enzyme E1 in the presence of ATP (Figure 1.14). The activated ubiquitin is then transferred to a Ub-conjugating enzyme E2 and eventually, is transferred to the target

substrate protein, in a process that typically involves an E3 Ub-ligase (Hershko et al., 1983).

When the ubiquitination cascade begins, an ubiquitin adenylate intermediate, in which ubiquitin and adenosine monophosphate (AMP) are conjugated by a high-energy thioester bond, is formed. This intermediate is then coupled to the E1 through a non-covalent bond. At this point the activated ubiquitin protein is transferred to a Cysteine residue on E1 and AMP is released. The E1-ubiquitin complex transfers the ubiquitin to an E2 enzyme through a transthioesterification reaction and the E1 ubiquitin-activating enzyme is then released.

Two ubiquitin specific E1 enzymes have been described in human, namely UBE1 and UBA6 (Jin et al., 2007). There are approximately 40 E2 enzymes in humans, which are characterized by the presence of an UBiquitin Conjugating (UBC) domain, of around 150 amino acids, which contains the catalytic Cysteine residue and interacts with the E1 (van Wijk and Timmers, 2010).

The E2-ubiquitin complex is then recognized by an ubiquitin ligase (E3), of which there are several hundred. They are divided in two major types: the catalytic Homologous to the E6-AP Carboxyl Terminus (HECT) domain E3s and the adaptor Really Interesting New Gene (RING) finger based E3s (Figure 1.14).

The HECT ligase is composed by two parts, in which the N-terminus interacts with the substrate and C-terminus contains the HECT domain. The HECT domain in turn has a C-terminal lobe that binds the ubiquitin and an N-terminal lobe, which is important for its interaction with the E2 conjugating enzyme (Huang et al., 1999). During conjugation, the ubiquitin molecule is first transferred to a Cysteine residue in the HECT sequence and then transferred to the Lysine of the substrate. The first member of the family to be discovered is the E6-Associated Protein (E6AP), which cooperates with the oncoprotein E6 in stimulating p53 ubiquitination and degradation (Talis et al., 1998).

The second family includes the RING E3 finger that has a zinc atom coordinated by Cysteine and Histidine residues (Lorick et al., 1999). RINGs have the ability to catalyse the direct transfer of ubiquitin from E2 ligases to the Lysine of the target proteins. In general, RING E3s can act as a single or a multiple component. The RING E3 MDM2, which is an example of a single component E3, has an important role in maintaining the low basal level of the tumor suppressor p53. As a matter of fact, MDM2 binds p53 and promotes its ubiquitination, leading to its proteasomal degradation (Haupt et al., 1997; Honda et al., 1997). When DNA damage occurs, MDM2 and p53 are phosphorylated and thus no longer able to interact. As a consequence, the level of p53 increased (Maya et al., 2001; Unger et al., 1999).

The Skp1/Cullin/F box protein (SCF) and Anaphase Promoting Complex (APC) are two members of the E3 family that act as multicomponent complexes and are involved in the regulation of the correct progression of the cell cycle. APC consists of more than thirteen subunits, whereas SCF consists of S-phase Kinase associated Protein 1 (SKP1), Cullin1, F-Box and a RING finger component Ring BoX protein1 (RBX1). Cullins are subjected to a process known as neddylation, where the ubiquitin-like protein NEDD8 is coupled to a conserved Lysine residue at its C-terminus (Osaka et al., 1998); the attachment of NEDD8 leads to a conformational change of SFC, in which RBX acquires enhanced catalytic activity (Kawakami et al., 2001). Moreover, cullin neddylation prevents the interaction with its inhibitor, Cullin-Associated and Neddylation-Dissociated 1 (CAND1)(Liu et al., 2002).

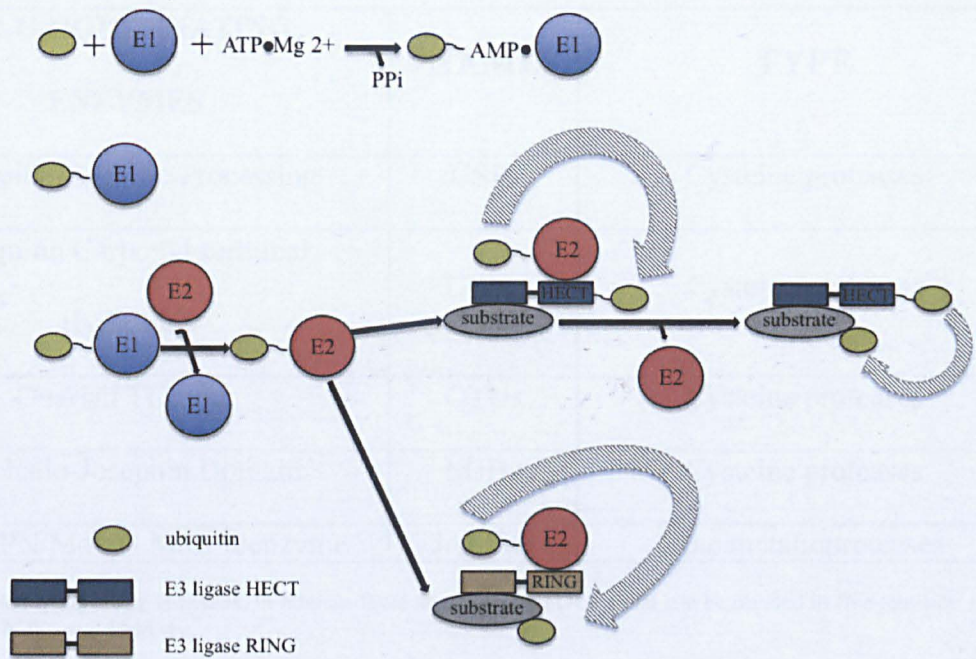


Figure 1.14. Ubiquitination pathway. Ubiquitination is a three-step enzymatic process. The first step consists of the ATP-dependent activation of ubiquitin, which is transferred to the Ub-activating (E1) enzyme. In this step there is the formation of a thioester bond between the C-terminal of Ub and the cysteine of the active site of E1. In the second step, ubiquitin is transferred from the E1 to the cysteine of E2 with a formation of a new thioester bond. In the third step, E2-ubiquitin complex is recognized by an E3 ligase. HECT type ligases bind the active ubiquitin and transfer it to a Lysine residue of the substrate protein. The RING finger-type ligases transfer the active ubiquitin directly from the E2 enzyme to the substrate protein. Adapted from (Kawabe and Brose, 2011).

1.1.2. De-Ubiquitination

De-ubiquitinating enzymes are a group of proteases that cleave the isopeptide bond on the carboxy-terminal side of G76 of ubiquitin thus releasing the ubiquitin linked to the protein. In this manner ubiquitination can be reversed without degrading the substrate protein. In humans there are around 100 DUBs that can be divided in five families namely USP, UCH, OTU/Cezanne, MJD, which are Cysteine proteases and JAMM, a zinc-dependent metalloproteases (Amerik and Hochstrasser, 2004), listed in Table 1.3.

DE-UBIQUITINATING ENZYMES	NAME	TYPE
Ubiquitin-Specific Processing	USPs	Cysteine proteases
Ubiquitin Carboxyl-terminal Hydrolase	UCHs	Cysteine proteases
Ovarian TUmour	OTUs	Cysteine proteases
Machado-Josephin Domain	MJDs	Cysteine proteases
Jab1/MPN/Mov34 Metalloenzyme	JAMMs	Zinc metalloproteases

Table 1.3. De-ubiquitylating enzymes. In humans there are sound 100 DUBs that can be divided in five families: USPs, UCHs, OTUs, MJDs and JAMMs.

As mentioned before, ubiquitin is translated as a long polymer, which possesses multiple ubiquitin subunits (Figure 1.15). DUBs enzymes, such as Ubiquitin Carboxy-terminal Hydrolase L1 (UCH-L1), process ubiquitin precursor in order to obtain ubiquitin monomers (Larsen et al., 1998). De-ubiquitination mainly serves to regulate the stability of the proteins that are targeted to proteasomal degradation and to maintain the homeostatic level of free ubiquitin in the cell. As a matter of fact, de-ubiquitination of p53 by the Herpesvirus-Associated Ubiquitin Specific Protease (HA-USP) protects p53 from proteasomal degradation (Li et al., 2002). DUBs can also act in exchanging two different types of poly-ubiquitin chains. One example is the OTU-type DUB A20, which is an inhibitor of NF-KB signalling. In fact A20 removes K63 ubiquitin chains from the Receptor Interacting Protein (RIP), which is a mediator of the proximal Tumor Necrosis Factor Receptor (TNFR1) signalling complex and binds K48 chains to RIP. Thus A20 acts as a negative regulator of NF-KB signalling targeting RIP for proteasomal degradation (Wertz et al., 2004).

At the level of the proteasome, poly-ubiquitin chains are released from the ubiquitin conjugated protein, thereby recycling ubiquitin (Verma et al., 2002; Yao and Cohen,

2002). This prevents both the degradation of ubiquitin and its interference with the protein entering into the proteasome. In Figure 1.15 are reported the general roles of DUBs.

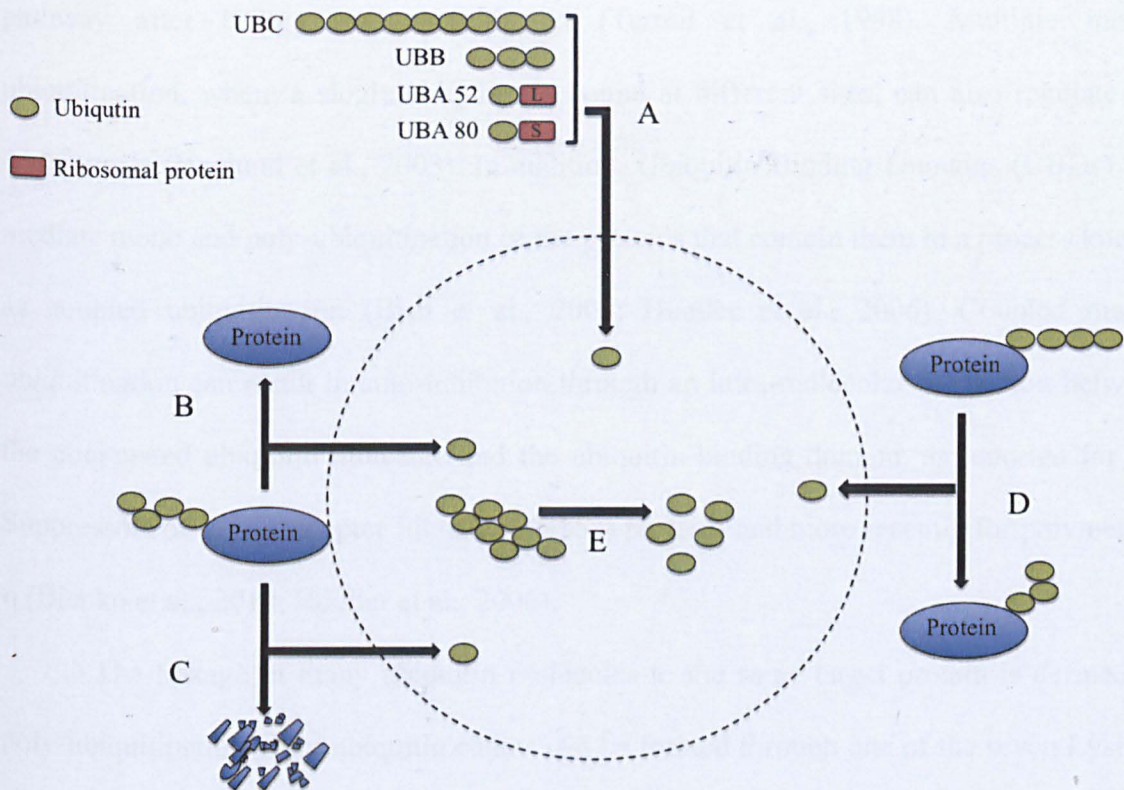


Figure 1.15. General roles of DUBs. A) The precursor of ubiquitin is translated as a fusion protein of multiple copies of ubiquitin. DUB must be cleaved at the K76 in order to obtain the mature form of the protein. B) DUBs avoid the degradation of the ubiquitinated protein. C) At the level of proteasome, DUBs remove poly-ubiquitin chains from the substrate protecting the poly-ubiquitin chain from degradation. D) DUBs remove poly-ubiquitin chains promoting the switching with other type of poly-ubiquitin chains. E) DUBs cleave the isopeptidic bond between poly-ubiquitin chains thus maintaining the homeostatic level of free ubiquitin pool. Adapted from (Komander et al., 2009).

1.1.3. Types of Ubiquitination

Ubiquitination acts as an important signalling molecule and the information that it transmits depends on the type and the nature of the ubiquitin chain (Table 1.4). As a matter of fact, proteins can be modified by mono-ubiquitination, which consists in the conjugation of a single ubiquitin molecule to one of the Lysines of the protein target and/or be poly-ubiquitinated, when further ubiquitin are attached to the ubiquitin monomer.

Mono-ubiquitination was first found in histones H2A (Goldknopf et al., 1975). It

was described as a mechanism for the regulation of the chromatin structure and gene expression (Zhang, 2003). In both yeast and mammals, it has also been found to regulate membrane receptors, which are down-regulated by internalization into the endocytic pathway after being mono-ubiquitinated (Terrell et al., 1998). Multiple mono-ubiquitination, where a single ubiquitin is bound at different sites, can also regulate the endocytosis (Haglund et al., 2003). In addition, Ubiquitin Binding Domains (UBDs) can mediate mono and poly-ubiquitination of the proteins that contain them in a process known as coupled ubiquitination (Bish et al., 2008; Hoeller et al., 2006). Coupled mono-ubiquitination can result in auto-inhibition through an intra-molecular interaction between the conjugated ubiquitin molecule and the ubiquitin-binding domain, as reported for the Suppressors of T-cell receptor Signaling (STSS) proteins and more recently for polymerase η (Bienko et al., 2010; Hoeller et al., 2006).

The linkage of many ubiquitin molecules to the same target protein is defined as poly-ubiquitination. Poly-ubiquitin chains can be formed through one of the seven Lysines present in the ubiquitin: K6, K11, K27, K29, K33, K48 and K63. These chains can be of different lengths and shapes and have been associated to different pathways (Table 1.4).

The canonical signal for proteasomal degradation is the K48 poly-ubiquitin chain (Chau et al., 1989) although K29 linked chains have also been found on target proteins toward this machinery (Johnson et al., 1995). It has been found that K11-linked chains, an abundant type of poly-ubiquitin chains in the cells, may act as a signal for proteasome degradation in the Endoplasmic Reticulum Associated protein Degradation (ERAD) pathway (Xu et al., 2009). In yeast it appears that the ultimate fate of all the chains, except K63, is proteasomal degradation (Xu et al., 2009).

Nevertheless, the other poly-ubiquitin chains, except K48, are also involved in non-proteolytic pathways and they play a role in processes including DNA repair and inflammation. K63 has been reported to be important in the DNA damage response. In

S.cerevisiae, mutation of the K63 renders the cell highly sensitive to UV irradiation (Spence et al., 1995). K63 chains also play a role in DNA damage tolerance, as described in the paragraph 1.3.1.2. (Hoegel et al., 2002). K63 chains also have an important role in NF- κ B activation by Tumor Necrosis Factor α (TNF α .) In this case, UBC13/MMS2 is paired with specific E3s to target different intermediates of these pathways. The TNF Receptor Associated Factor 2 (TRAF2) E3, for examples, mediates the poly-ubiquitination of the Receptor Interacting Protein (RIP) during TNF α signalling and these chains are then recognized by the ubiquitin binding protein NF-kappa-B Essential MOdulator (NEMO), which induces the activation of the I κ B Kinase (IKK) (Ea et al., 2006; Li et al., 2006; Wertz et al., 2004; Wu et al., 2006).

Besides these pathways, the roles of other types of poly-ubiquitin chains have been reported and poly-ubiquitin chains with mixed linkage have also been described. For example K29/K33 linked mixed chains have been described in the regulation of AMP-activated protein Kinase (AMPK)-related kinases (Al-Hakim et al., 2008). Linear poly-ubiquitin chains are a particular type of poly-ubiquitin chains, which are assembled by the E3 ligase known as the Linear UBiquitin chain Assembly Complex (LUBAC); these types of chains are generated through the linkage between the N-terminal Methionine of one ubiquitin and the C-terminal Glycine of another ubiquitin molecule and have been shown to be involved in the NF- κ B activation (Kirisako et al., 2006; Tokunaga et al., 2009).

CHAIN	ROLE(S)
K6	DNA repair
K11	ERAD
K27	?
K29	Degradation
K33	?
K48	Degradation
K63	DNA repair, damage tolerance, inflammation

Table 1.4. Types of poly-ubiquitin chains. Ubiquitin monomers can be assembled to form poly-ubiquitin chains, which are formed through the seven internal Lysine residues. Different poly-ubiquitin chains are associated to different pathways. Adapted from (Wong and Cuervo, 2010).

1.1.4. Proteasomal Degradation

The main function of the proteasome is to degrade ubiquitin targeted proteins by proteolysis (Figure 1.16). It is present inside all eukaryotes, archaea and in some bacteria and is located in both the nucleus and cytoplasm (Peters et al., 1994).

The proteasome is a cylindrical complex, which consists of one 20S core particle, capped at one or both ends by a 19S complex, known as the 19S regulator complex (Peters et al., 1994) (Figure 1.16). The 20S subunit is composed of four rings around a central pore. Each ring contains seven subunits and the two inner rings contain 7 β -type proteolytical subunits, while the external ones are formed by 7 α -type subunits, acting as a gate through which proteins can enter (Groll et al., 1997). The 19S subunit is involved in the recognition and unfolding of poly-ubiquitinated substrates and in opening the 20S gate (Smith et al., 2007). The 19S particle is formed by two subunits: the base and the lid (Glickman et al., 1998). The base contains ten subunits, of which six are ATPase subunits

(RPT1-RPT6) and four are non-ATPases (RPN1, RPN2, RPN10, RPN13). The lid has nine subunits among which there is the de-ubiquitylating enzyme RPN11 (Glickman et al., 1998; Verma et al., 2002). Ubiquitinated proteins can be directly recognized by RPN10/S5a and RPN13/Adrm1 receptors of the base 19S of the proteasome or can be escorted to the proteasome by the shuttle factors, like the Radiation Sensitive 23 (RAD23), which bind the substrate through their UBiquitin-Associated (UBA) domain and interact with proteasome through its UBiquitin-Like (UBL) domain (Deveraux et al., 1994; Husnjak, Elsasser et al. 2008; Chen and Madura, 2002). These proteins recognize the ubiquitinated substrates and drive them into the proteasome. Proteins first pass through the translocation channel of the 19S subunit. Proteins with folds that are too large to enter channel are unfolded by ATPase activity of the 19S subunit (Wenzel and Baumeister, 1995). The ubiquitin linked to the protein is removed by the proteasome associated DUB subunits. In this way, ubiquitin can be recycled and it does not interfere with the entrance of the protein in the proteasome (Verma et al., 2002). Together the DUBs and unfolding activities allow the protein to enter the proteasome, where the mechanism of proteolysis is carried out by the β subunits of the 20S core particle in a trypsin-like and chymotrypsin-like fashion (Heinemeyer et al., 1997).

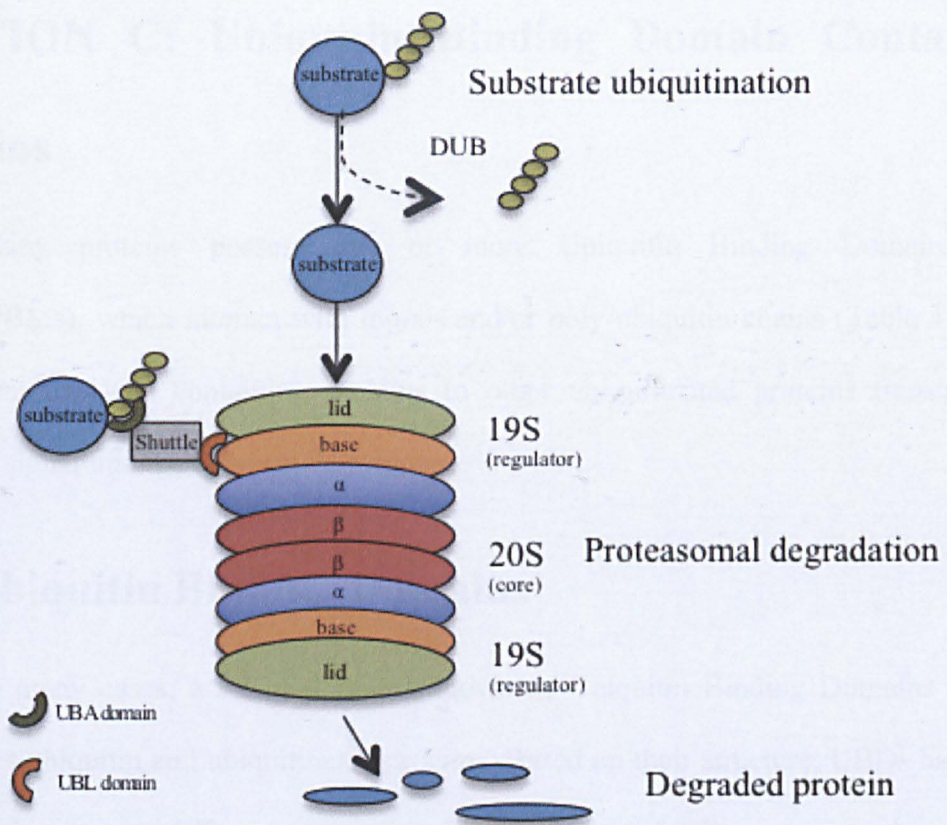


Figure 1.16. Proteasomal degradation. Proteasome is composed by 20S core and 19S regulator particles. 20S particle is composed by four rings: two α gates and two β proteolytic subunits. Ubiquitinated protein are recognized directly by the receptors of the base 19S or escorted by shuttle factors which bind the substrate through the UBA domain and interact with proteasome by UBL domain.

SECTION C: Ubiquitin Binding Domain Containing

Proteins

Many proteins possess one or more Ubiquitin Binding Domains/Motifs (UBDs/UBMs), which interact with mono- and/or poly-ubiquitin chains (Table 1.5). The recruitment of UBD containing proteins to other ubiquitinated proteins translates the ubiquitin signal into the appropriate response.

1.1. Ubiquitin Binding Domains

In many cases, a set of domains known as Ubiquitin Binding Domains (UBDs) recognizes ubiquitin and ubiquitinated proteins. Based on their structure, UBDs have been classified into twenty different groups, in which the largest family comprises domains that interact with ubiquitin through a α -helix, such as the Ubiquitin Associate (UBA) and the Ubiquitin Interacting Motif domain (UIM) (Table 1.5). The second largest group comprises UBDs that have a zinc finger in their sequence, such as the Npl4 Zinc Finger domain (NZF) and the Ubiquitin Binding Zinc finger domain (UBZ). UBDs are listed in Table 1.5 (panel A). So far, five UBDs are known to be involved in DNA damage repair: the UBA (Hofmann and Bucher, 1996), UIM (Hofmann and Falquet, 2001), the Ubiquitin E2 Variant (UEV) (Koonin and Abagyan, 1997; Ponting et al., 1997), the Ubiquitin Binding Motif (UBM) and UBZ domains (Bienko et al., 2005). For example, the Receptor Associated Protein 80 (RAP80), which is involved in the recruitment of BRCA1 to sites of DNA damage, contains two IUMs (Yan et al., 2002). RAP80 UIMs preferentially binds K6 and K63 poly-ubiquitin chains, which are synthesized at the damage sites (Sobhian et al., 2007).

The UBZ domain was first described in the Y-family DNA polymerases η and κ (Bienko et al., 2005) and have been reported in other proteins such as WHIP (Bish and

Myers, 2007) and RAD18 (Notenboom et al., 2007). Between all the UBZ domains that are listed in Table 1.5, (panel B), UBZ4, which is a C2HC zinc finger domain, has been found in Ubz1 (also called Spartan or DVC1) protein (Centore et al., 2012), and this protein is the major subject of my thesis work.

A

Symbol	Name	Protein examples
UBA	UBiquitin Associated	RAD23
UIM	UBiquitin Interacting Motif	RAP80, Ataxin 3
UBZ	Ubiquitin Binding Zinc finger	RAD18, WHIP
UBM	Ubiquitin Binding Motif	REV1
GLUE	GRAM Like Ubiquitin binding in EAP45	EAP45
NZF	Npl4 Zinc Finger	Npl4
DUIM	Double sided Ubiquitin Interacting Motif	HRS
MIU	Motif Interacting with Ubiquitin	Rebex5
GAT	GGA and TOM	GGA, TOM
PFU	PLAA Family Ubiquitin binding domain	PLAA
VHS	Vps27/Hrs/STAM	STAM1
SH3	Src Homology 3	Cin85
CUE	Coupling of Ubiquitin conjugation to Endoplasmatic reticulum degradation	SMRCD
UEV	Ubiquitin E2 Variant	MMS2

Symbol	Protein examples	Structure
UBZ1	TAX1BP1	C2H2
UBZ2	FLJ44922	C2HC
UBZ3	Polymerase η	C2H2
UBZ4	Polymerase κ , WHIP, Rad18, Ubz1	C2HC

Table 1.5. Ubiquitin binding domains. The table in panel A shows many of UBDs and some examples of proteins where they have been found. In table in panel B are listed the UBZ domain types, their structure and examples of protein where they have been found.

1.2. Ubiquitin Binding Proteins

Proteins that contain the Ubiquitin Binding Zinc finger domain 3 and 4 (UBZ3/UBZ4) have been found to be involved in DNA replication and repair pathways. Six proteins containing UBZ 3 and 4 domains have been reported: RAD18 (Notenboom et al., 2007), WRNP1/WHIP (Bish and Myers, 2007), polymerase η and κ (Bienko et al., 2005), the Sensitive to Nitrogen Mustard 1 A (SNM1A) (Yang et al., 2010) and C1ORF124 (Spartan/DVC1/Ubz1) (Centore et al., 2012) (Figure 1.17).

All of these proteins are involved in DNA damage repair; RAD18, polymerase η and κ participate in DNA damage tolerance and SNM1A in the repair of DNA interstrand crosslinks (Hoege et al., 2002; McDonald et al., 1997; Ulrich and Jentsch, 2000; Wagner et al., 1999; Yang et al., 2010). The role of WHIP remains unclear and most of the studies on molecular function have been done on the yeast homologue MGS1. It has been reported that MGS1 is required for genome stability of budding yeast and that it blocks the activity of the RAD6 pathway in the absence of an exogenous DNA damage, via interactions with PCNA (Hishida et al., 2001; Hishida et al., 2002; Hishida et al., 2006).

UBZ3/4 domains are important in directing the protein to the site of DNA damage. A single point mutation in the UBZ domain of the two Y-family polymerases impedes their binding to mono-ubiquitinated PCNA; as a consequence, after DNA damage, mutant pol η and κ are not localized to nuclei foci and cannot participate in TLS (Bienko et al., 2005). Similarly, the UBZ domain of RAD18 is also required for the protein to migrate to the sites of DNA damage (Nakajima et al., 2006).

More recently it has been demonstrated that the SNM1A protein, which is required for cellular processing of inter-strand DNA crosslinks, also possesses a conserved UBZ domain, which appears to be important in binding mono-ubiquitinated PCNA. Also in this case, DNA damage drives SNM1A to nuclear foci in a UBZ-dependent manner (Yang et al., 2010).

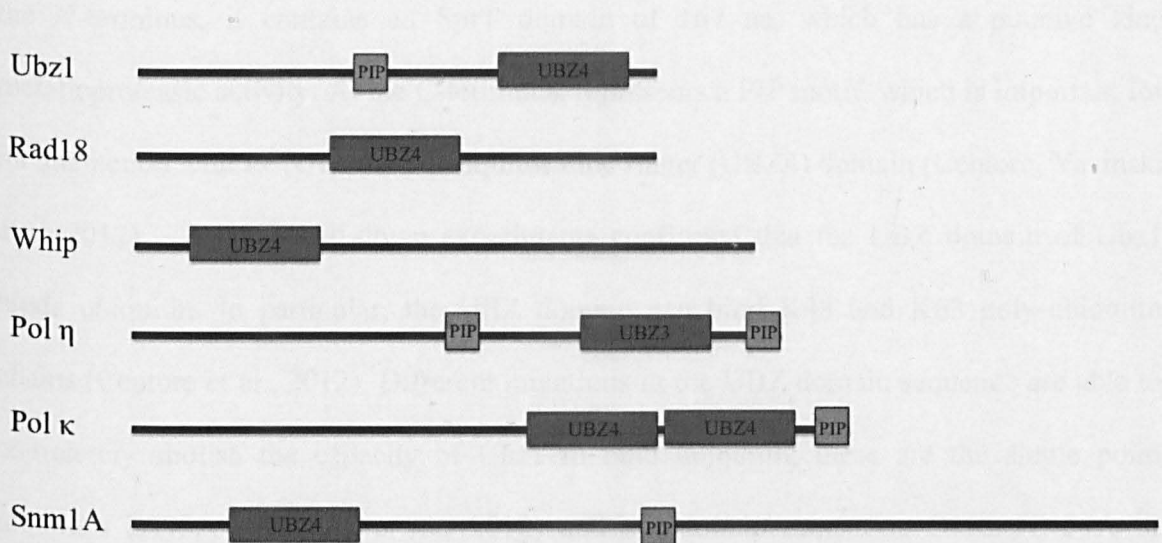


Figure 1.17. UBZ containing proteins. UBZ domains are reported as UBZ3 (C2H2) or UBZ4 (C2HC) zinc finger. Ubz1, RAD8, WHIP, POL κ and SNM1A contain a UBZ4 domain. UBZ3 has been found only in POL η . The main difference between the two domains is the forth ligand, which is a Histidine in UBZ3 and a Cysteine in UBZ4. All of these proteins are involved in DNA damage repair pathways.

1.2.1. C1orf124 (Spartan/DVC1/Ubz1)

K6 poly-ubiquitin chains have been found to be involved in DNA damage-related signalling pathways (Wu-Baer et al., 2003). In order to identify proteins that bind K6 poly-ubiquitin chains, my group performed a proteomic screen. The top candidate for the interaction with the K6 poly-ubiquitin chain was WHIP and the ubiquitin interacting sequence was found to be Rad18-like zinc finger, which was a *bona fide* Ubiquitin Binding Zinc finger domain (UBZ) (Bish and Myers, 2007). Using a database search for UBZ domains, we found six human proteins with putative UBZ domains: RAD18, WHIP, polymerase η and κ , SNM1A, and the uncharacterized protein C1ORF124, renamed Ubz1/SPARTAN/DVC1.

Ubz1 is a 489 aa protein of 55 kDa, which is encoded by the C1ORF124 gene. At the N-terminus, it contains an SprT domain of 167 aa, which has a putative zinc metalloprotease activity. At the C-terminus, it presents a PIP motif, which is important for its interaction with PCNA, and a Ubiquitin Zinc finger (UBZ4) domain (Centore, Yazinski et al. 2012). *In vitro* pull-down experiments confirmed that the UBZ domain of Ubz1 binds ubiquitin. In particular, the UBZ domain can bind K48 and K63 poly-ubiquitin chains (Centore et al., 2012). Different mutations in the UBZ domain sequence are able to completely abolish the capacity of Ubz1 to bind ubiquitin; these are the single point mutation D473A (Machida et al., 2012) and the double mutations C456A/C459A or C456G/C459G (Davis et al., 2012; Mosbech et al., 2012; Centore et al., 2012).

As has been previously described, ubiquitination of PCNA appears to act as a switch that controls the decision between S phase specific DNA repair pathways. The UBZ domain has a role in directing proteins to the site of DNA damage via its ability to bind to ubiquitinated PCNA. This role has been proposed because the UBZ containing proteins polymerase η and κ and RAD18 are all found in DNA damage induced nuclear foci and their localization requires a functional UBD (Bienko et al., 2005; Crosetto et al., 2008;

Nakajima et al., 2006). Many UBZ-containing proteins do not interact with PCNA exclusively through their UBZ domains, because they also possess a PIP motif (Warbrick et al., 1995); the PIP box motifs are involved in the interaction between PCNA and three UBZ containing proteins: polymerase η (Haracska et al., 2001) and polymerase κ (Haracska et al., 2002) and SNM1A (Yang et al., 2010). Other proteins interact with PCNA even if they do not have a PIP box motif; for example, RAD18, which is involved in PCNA ubiquitination, has been found to have PCNA binding motif in its N-terminal region, which does not match with the PIP box motif (Notenboom et al., 2007). Thus, the UBZ domain and the PIP box motif work together in mediating the physical interaction between these UBZ containing proteins and PCNA.

The PIP box of Ubz1 is an 8 amino acids sequence, which has an important role in the interaction with the PCNA. *In vitro* pull-down experiments showed that deleting the entire motif (Ghosal et al., 2012), or mutations of residues Q325A, L328A, Y331A, and F332A (Davis et al., 2012) or even of just Y331A and F332A (Machida et al., 2012; Mosbech et al., 2012), completely abolish the capacity of Ubz1 to bind PCNA.

The SprT domain carries a conserved metalloprotease HExH motif, which is found in many zinc metalloproteases (Rawlings and Barrett 1995; Jongeneel et al., 1989). In the SprT domains, the zinc ion is coordinated by two Histidine residues. A Glutamic acid and a molecule of water work together in the cleavage of the isopeptidic bond (Kim et al., 2013; Rawlings and Barrett, 1995). However, no eukaryotic proteins with this domain have been functionally characterized.

The goal of my thesis has been to better characterize Ubz1. In particular, I investigated the interaction between Ubz1 and PCNA using SprT and UBZ domain mutant constructs. In addition, Ubz1 appeared to be subjected to an SprT-dependent autocleavage and my results suggested that the SprT domain has a metalloprotease function.

2. METHODS

2.1. Solutions

- PBS buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM NaH₂PO₄, 2mM K₂HPO₄, pH 7.4
- TBS buffer: 50 mM Tris base, 150 mM NaCl, pH 7.4
- TTBS buffer: 1x TBS, 0.1% Tween-20
- BBS 2x: 50 mM Bes, 280 mM NaCl, 1.5 mM Na₂HPO₄, pH 7
- LB medium (Luria-Bertani medium): 10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L, pH 7.5-8)
- TB (Terrific-Broth): 12 g tryptone, 24 g yeast extract, 9.4 g K₂HPO₄, 2.2 g KH₂PO₄, 4 mL glycerol in 1 L
- LYSIS BUFFER: 50 mM Hepes pH 7.4, 150 mM NaCl, 1 mM EDTA, 0,1% NP40
- UREA BUFFER: 8 M urea, 10 mM Tris-HCl, 115 mM NaH₂PO₄, 300 mM NaCl, and 0.1% NP40, pH adjusted to 8.0 with NaOH
- BLUE COMASSIE: 10% (NH₄)₂SO₄, 0.03% Comassie BB G250, 2.5% H₃PO₄, 20% Ethanol
- 0.2 M IMIDAZOLE
- 0.1% SDS
- 0.2 M ZINC SULFATE
- DI-AP (DIAMMONIUM PHOSPHATE) pH 8
- 50% MEOH 20 mM di-AP
- 200 mM IODOACETAMIDE
- 5% FORMIC ACID
- 100% ACN (Acetonitrole)
- 0.2% TFA (Trifluoroacetic acid)
- 50% ACN/50% TFA 0.2%
- H₂O/ 10% glycerol
- 2.5M CaCl₂
- 2x LAEMMLI BUFFER: 4% SDS, 20% glycerol, 0.125 M Tris HCl, 0.004% Bromophenol blue, 10% beta-mercaptoethanol, pH 6.8
- NP40 BUFFER: 1X PBS, 140 mM NaCl, 1% NP40
- TCEP: tris(2-carboxyethyl)phosphine

2.2. DNA and Cloning

2.2.1. Plasmids Construction

The pHA-MAT derives from the pCGN vector and its construction has already been described (Bish et al., 2008). Full length Ubz1 was cloned from a sequence verified cDNA MHS1010-7508511 (Open Biosystems) by PCR into pHA-MAT. The D473A, H111L/E112A, E112A Ubz1 mutants were generated using the site-directed mutagenesis. The pCGT7 vector derived from the pCG vector in which the T7 tag (MASMTGGQQMG) was inserted. The full length Ubz1 was inserted by ligation into the pCGT7. The PCNA, Ub-PCNA and 4Ub-PCNA constructs, synthesized by Genescript, were digested with XbaI and BamHI and ligated into pHA-MAT and pCGT7 using standard techniques.

2.2.2. Site Directed Mutagenesis

PCR- In order to build pHA-MAT-Ubz1 D473A, E112A, H111L/E112A mutants, pHA-MAT-Ubz1 WT was used as a template. The mix was composed by: 20 ng DNA, 1x ThermoPol buffer (NEB), MgSO₄ 4 mM, dNTPs mix (200 μM), 250 nM oligonucleotides primers.

Primers:

- pHA-MAT-Ubz1 (E112A):

FORWARD 5'-AGACCCTCCTGCATGCAATGATACATGCCTA
REVERSE 3'-TAGGCATGTATCCATTGCATGCAGGAGGGTCT

- pHA-MAT-Ubz1 (H111L/E112A):

FORWARD 5'-AGACCCTCCTGCTTGCAATGATACATGCCTA
REVERSE 3'-TAGGCATGTATCCATTGCAAGCAGGAGGGTCT

Cycling parameters for the quickchange site directed mutagenesis:

SEGMENT	CYCLES	TEMPERATURE	TIME
1	1	95°C	30 sec
2	18	95°C	30 sec
		55°C	1 min
		68°C	1 min/kb (circa 6,5 min)

Table 2.1. PCR cycles of site directed mutagenesis.

DNA DIGESTION- At the end of the reaction, samples were kept in ice for 2 minutes to be cooled and the template plasmids were digested via the addition of NEB Buffer 4 (to 1x) and 1 µL of DpnI. The reaction was incubated for 1h at 37°C.

TRANSFORMATION- 2 µL of the digestions were electroporated in GC10TM electrocompetent cells at 1500 V and resuspended in 960 µL of LB medium. The transformation was incubated 1h at 37°C and 40 µL of the reactions were plated on the agar plate, supplemented with the antibiotic.

ELECTROCOMPETENT CELLS- 40 mL of GC10TM cells were grown overnight at 37°C. The day after they were diluted 1/10 into 400 mL LB and growth until OD₆₀₀ ~ 0.75 and chilled on ice. Cells were pelleted at 4000x g at 4°C for 15 minutes and then resuspended in 400 mL sterile ice-cold water. Cells were pelleted again at 4000x g for 15 minutes and resuspended in 200 mL sterile cold water. This step was repeated four times and at the end cells were resuspended in 800 µL sterile cold water/ 10% glycerol and aliquoted before storing at -80°C.

DNA SEQUENCING- DNA was prepared using the Qiagen mini-prep kit and the entire cDNA was sequenced by “BMR Genomics”, using 300 ng of DNA and 6.4 pmol of primer. The presence of the mutations were confirmed using the “4Pics” program.

2.3. Cells handling

2.3.1. Cell Culture

HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/mL penicillin, 100 µg/mL streptomycin B, 25 ng/mL amphotericin B.

2.3.2. Transfection

Plasmids were purified from GC10 *E.coli* using GenEluted™ HP Endotoxin-Free Plasmid Maxiprep Kit. Plasmids have been transfected into HEK293T cells using the calcium phosphate transfection method. Cells were plated the day before transfection in order to have a confluence of 40% the day of the transfection. The mix used for the transfection of a 10 cm dish was composed by: 60 µL CaCl₂ 2.5 M, 10 µg DNA and H₂O to final volume of 500 µL and 500 µL of 2x BBS was added drop wise while agitating the tube. The resulting mixture was then added over the cells and incubated for 24 hours. The transfection mix was removed and the cells washed 1x with PBS and returned to their growth media.

2.4. Protein Purification

2.4.1. Lysates Collection

Two days after transfection, cells were washed twice with ice cold PBS. Lysates were resuspended in the lysis buffer, sonicated in 0.5 seconds bursts for 1 minute at an

amplitude of 60% using a Hielscher sonicator fitted with a voltmeter adapter, and centrifuged 10 minutes at 13000 rpm. Bradford assay method (Sigma Aldrich) was used in order to measure the protein concentration in the lysates collected. The manufacturer's instructions were followed. Laemmli Buffer 2x (Sigma) was added to the lysates and samples were boiled for 5 minutes.

2.4.2. Immunoprecipitation

Agarose beads (ezviewTM Red Anti-HA Affinity gel, Sigma-Aldrich or T7-Tag antibody agarose beads, Novagen), were equilibrated in the lysis buffer. Lysates were transferred to the beads and kept rotating for 1h at 4°C. Immune-complexes were washed three times with lysis buffer, transferred in new tubes then washed twice with the PBS supplemented with 0.1% NP40. 2x Laemmli Buffer (Sigma) was added and samples were boiled.

2.4.3. Peptide Elution

After the immunoprecipitation with the beads, protein complexes were eluted 3x for 20 minutes with a HA peptide in lysis buffer (200µg/mL final concentration). Samples were transferred in the speedvac. Laemmli Buffer 2x (Sigma) was added and samples were boiled.

2.4.4. Denaturing Tandem Affinity Purification

After the 1-hour IP, beads were washed three times with the lysis buffer. For urea elution, 2 x 200 µL of urea buffer was added directly to the antibody-conjugated beads, and the supernatant was removed following three minutes incubation at room temperature. The supernatant was then incubated with the Ni-NTA beads (Sigma Aldrich) (pre-equilibrated with urea buffer) at room temperature for 1 hour. The beads were washed 4 times with the urea buffer and three times with the lysis buffer. Laemmli Buffer 2x (Sigma) supplemented with 1mM TCEP and 33 mM EDTA was added and the samples were boiled for 8 minutes.

2.5. Protein Visualization

2.5.1. Protein Staining

COLLOIDAL BLUE COMASSIE STAINING- Gels were stained with the colloidal blue Comassie for at least 1 hour and then rinsed with water.

ZINC STAINING- Zinc stain deposits a zinc metal precipitate in the gel. The SDS coating on the proteins prevents the stains from binding to the proteins, while the gel turns white. SDS-PAGE gels were put on an orbital mixing platform in the Imidazole solution for 10 minutes and then in the Zinc sulphate solution for 30 seconds. The gel was rinsed with DDI water and put on a black background in order to see the bands.

2.5.2. Western Blotting

Samples in the 2x Laemmli sample buffer (Sigma Aldrich) were heated at 100°C for 8 minutes, centrifuged and run in SDS-PAGE agarose gels. Proteins were transferred to a PVDF membrane (Millipore) using an electroblotting procedure. Membranes were blocked for 1h in 5% milk (TTBS) and then incubated with the antibodies, diluted in blocking solution, for different times. Membranes were washed with TTBS and incubated for 1h with HRP conjugated secondary antibodies, diluted in 5% milk (TTBS). The presence of the proteins was revealed using Enhanced ChemiLuminescence (ECL). Antibodies are listed in Table 2.2.

PRIMARY ANTIBODIES	
α - HA HRP (clone HA-7, Sigma-Aldrich)	
α -T7 (Millipore)	
α -PCNA (clone IG7, Sigma-Aldrich)	
α -ubiquitin (clone P4D1, Cell Signalling)	
α -ubiquitin biotin conjugated (clone P4D1, Abcam)	
α -GAPDH HRP (clone 7.1.1., Sigma-Aldrich)	
α -C1orf124 (clone E6, D3, GenScript)	
α - γ H2AX (clone JBW301, Millipore)	

SECONDARY ANTIBODIES	
α - Mouse IgG- HRP (Sigma-Aldrich)	
α - Rabbit IgG-HRP (Cell Signalling)	

Table 2.2. Antibodies used in this project.

2.5.3. Mass Spectrometry

SAMPLE PREPARATION FROM SDS-PAGE- Bands were cut from acrylamide gel and supplemented with 50 mM Ethylenediaminetetraacetic Acid EDTA (ph 8.0) for 15 minutes. Samples were then reduced with 2.5 mM DTT in 20 mM DiAmmonium Phosphate pH 8.0 (di-AP), and incubated at 60°C for 30 minutes. Followed by alkylation with 200 mM iodoacetamide for 1 hour in the dark. Gel slices were washed 3 times for 20 minutes with 1 mL 50% MeOH containing 20 mM di-AP. To dry the pieces, 100 μ L of 100% ACN was added. At the end, all dried slices were soaked with 10 μ L of Trypsin (10 ng/ μ L in 20 mM di-AP) for 25 minutes at 37°C. When the trypsin was absorbed, pre-warmed di-AP was added just to cover the gel. Samples were kept overnight at 37°C.

SAMPLE PREPARATION FROM BEADS - After the IP or the DTAP, the beads were washed three times with PBS and trypsin (10 ng/ μ L) was added to just cover the beads. Samples were incubated at 37°C for 6 hours. Supernatants were removed and transferred to new tubes. Beads were washed twice with PBS and pooled together. In addition 10 μ L of trypsin was added and the samples were incubated for 1 hour at 50°C.

DIGEST CLEAN UP PROTOCOL -After the trypsin digestion, samples were purified using the stage-tip method (Rappsilber et al., 2003). Digested supernatants were placed in new tubes. 5% of Formic Acid was added to the remaining gel pieces and the samples were sonicated for 15 minutes. The filter of the zip tips were wetted with 100% ACN and then 10 μ L of 0.2% TFA was passed through the filters. Then the samples were pushed through the column tips. Filter were washed with 50 μ L of 0.2% TFA and samples were eluted with 10 μ L of a mix composed of 50% ACN and 50% TFA 0.2%.

MASS SPECTROMETRY ANALYSIS – Samples were analysed with an ion trap mass spectrometer (Bruker). This massspectrometer uses an electrospray ionization (ESI) source coupled to a Proxeon NANO LC system. Gradient were developed using ACN from 1% to 50% over a 90 minute period. LC-MS/MS spectra were searched against the human protein database by the GPM interface to the X!Tandem algorithm (Craig et al., 2004).

3. AIM OF THE PROJECT

Ubz1 is the least characterized UBZ containing protein and recently it has been demonstrated to be involved in DNA repair. To further characterize Ubz1 I decided to organize this thesis into two parts. In the first half of my thesis I describe the Ubz1 post-translational modifications and its SprT domain-dependent autocleavage. Specifically, I have used an approach based on mass spectrometry coupled with western blotting analysis to evaluate Ubz1 ubiquitination and to analyze peptides derived from Ubz1 degradation. In the second part of my work, I evaluated the Ubz1/PCNA interaction by the creation of three PCNA constructs, which expressed PCNA unmodified or fused with mono- or tetra-ubiquitins. I made use of site directed mutagenesis in order to obtain Sprt and UBZ domain mutants, which were then used in many of these experiments.

4. RESULTS

4.1. Ubz1 Characterization

4.1.1 Ubz1 Post-translational Modifications

Ubiquitination is a post-translational modification that involves the carboxy-terminal Glycine of ubiquitin and the side chain of a Lysine of the target protein. Ubiquitin monomers can be assembled to form poly-ubiquitin chains. One feature of the UBZ family of proteins is that they can undergo UBZ-dependent ubiquitination. Therefore, UBZ domain containing proteins are both non-covalently bound to ubiquitin through their ubiquitin binding domains and are subjected to a covalent ubiquitination. This ubiquitination requires an intact ubiquitin-binding domain, in a phenomenon known as coupled ubiquitination (Bish et al., 2008; Hoeller et al., 2006).

Since Ubz1 has a UBZ domain (residue 453-476), I thought that it would also be subject to coupled ubiquitination. In order to test this feature, I performed a Denaturing-Tandem Affinity Purification (DTAP) of wild type Ubz1 and a version with a mutant UBZ domain. As reported for WHIP, the Alanine substitution of the highly conserved Aspartate in the UBZ domain abolishes the ubiquitin binding (Bish et al., 2008; Hoeller et al., 2006). These Ubz1 constructs have a double tag, which consists of a HemAgglutinin (HA) epitope followed by a Metal Affinity Tag (MAT). DTAP consists of two-step purification: first a non-denaturing immunoprecipitation, which is based on the HA antibody linked to agarose beads. This is followed by a second step of denaturing purification, in which the proteins are released from the HA-antibody by the addition of a chaotropic agent, such as urea. Subsequently, Ni-NTA beads are incubated with the eluted material, where the immobilized nickel binds the MAT tag (Bish et al., 2008) (Figure 4.1). I over-expressed

HA-MAT-Ubz1 WT and D473A Ubz1 mutant in HEK293T cells and I performed DTAP purification 48 hours post-transfection.

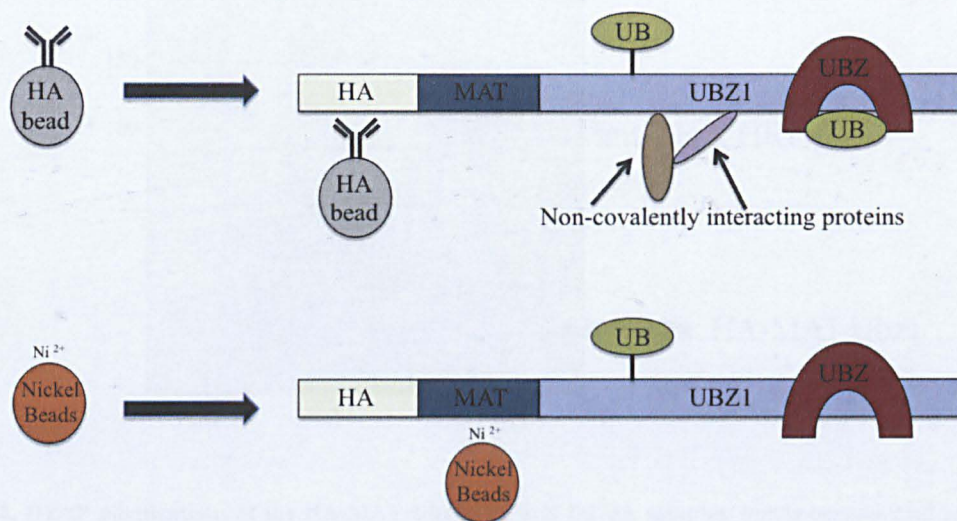


Figure 4.1. Denaturing Tandem Affinity Purification procedure (DTAP). The DTAP protocol consists of two steps: an immunoprecipitation against an epitope tag under non-denaturing conditions, followed by a denaturing purification using a Ni-NTA matrix, which binds a tandem metal affinity tag. The goal of this purification is to obtain proteins from their native environments with intact post-translational modifications in the absence of non-covalently interacting protein partners.

Subsequently, the DTAP purifications were analysed by western blot and I saw that the anti-HA blot of the over-expressed Ubz1 WT appeared as a smear (Figure 4.2, lane 2), while the D473A was a single band (Figure 4.2, lane 3). In particular, both HA-MAT-Ubz1 ran between 50 and 75 kDa and the percentage of unmodified Ubz1 WT was greater than 85%.

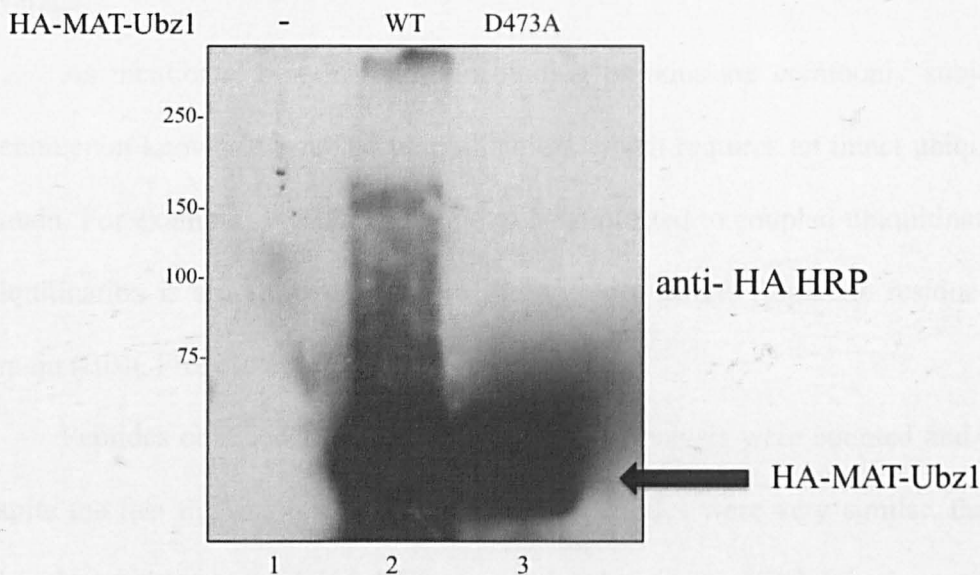


Figure 4.2. DTAP purifications of the HA-MAT-Ubz1 WT and D473A samples. Immunoprecipitated samples were run on a gel and blotted with the anti-HA HRP antibody. Wild type Ubz1 ran as a smear whereas the D473A mutant ran as a unique band.

Given these results, I thought that the smear might correspond to the ubiquitinated form of Ubz1 and to gain further insights into the ubiquitination of the wild type Ubz1, I blotted the same samples with a anti-ubiquitin antibody, but I was not able to obtain a convincing blot. Therefore, I decided to make use of mass spectrometry analysis to identify the ubiquitinated peptides in the Ubz1 purifications. To do this, I over-expressed HA-MAT-Ubz1 WT and D473A in HEK293T cells and after 48 hours, lysates were collected and Ubz1 was immunoprecipitated using anti-HA beads. In order to elute the specifically bound proteins, I incubated the bead bound immune-complexes with a high concentration of HA peptide diluted in lysis buffer. The HA peptide competes for the binding to the immobilized antibody, allowing the release of the HA-immune-complexes. I analyzed a portion of the samples by SDS-PAGE and visualized the eluted proteins by zinc staining. Finally, I processed the bands for analysis by LC-MS/MS. The LC-MS/MS analysis has a key weakness in that the mass spectrometer almost always undersamples the actual peptide content. In this case, I have summed the data from three runs in order to maximize the

coverage.

As mentioned before, ubiquitin binding proteins are commonly subjected to the phenomenon known as coupled ubiquitination, which requires an intact ubiquitin-binding domain. For example, WHIP was found to be subjected to coupled ubiquitination and this ubiquitination is strictly dependent on the presence of the Aspartate residue in its UBZ domain (Bish, Fregoso et al. 2008).

Peptides obtained from mass spectrometry analysis were counted and I found that despite the fact that the total number of Ubz1 peptides were very similar, the number of ubiquitin peptides in the Ubz1 WT was greater than in the UBZ domain mutant (D473A) sample (Table 4.1). This result, together with the data from the western blot analysis, suggests that Ubz1 might subjected to coupled ubiquitination, because this ubiquitination is strictly dependent on its UBZ domain. Thus my data are consistent with previous results, which also reported that the substitution of Aspartate at position 473 in UBZ domain of Ubz1 abolishes the ubiquitin binding (Machida et al., 2012).

Ubz1 TYPE	TOTAL Ubz1	UNIQUE Ubz1	TOTAL Ub	UNIQUE Ub
WT	2212	186	34	11
D473A	2043	200	5	3

Table 4.1. Ubiquitin peptides from HA-MAT-Ubz1 WT and D473A purifications. HA-MAT-Ubz1 WT and D473A were purified with the HA beads and eluted with the HA peptide. Immune-complexes were then digested with the trypsin and peptides were analysed by LC-MS/MS. Here the total and unique peptides for ubiquitin found in the analysis are reported. Total=total number of peptides identified. Unique peptide=a peptide with unique individual amino acids sequence is counted a single time regardless of how many times the mass spectrometer detected it. Here I report the number of peptides identified with a unique amino acids sequence for Ubz1 and ubiquitin.

At this point, I decided to blot the eluted HA-MAT-Ubz1 WT and D473A with the anti-ubiquitin antibody and I was able to detect the smear of ubiquitinated Ubz1 WT (Figure 4.3, lane 2), which I could not see in the DTAP purification. As reported in Figure

4.3 (lane 3, long exposure), only a weak band corresponded to mono-ubiquitinated Ubz1 was visible in UBZ domain mutant, indicating that the mutant was ubiquitinated at a much lower level than WT (lane 2), confirming the results shown in the blot in Figure 4.2. The anti-HA HRP blot in Figure 4.3 (lower panel, lane 2) showed that the HA-MAT-Ubz1 WT was ubiquitinated.

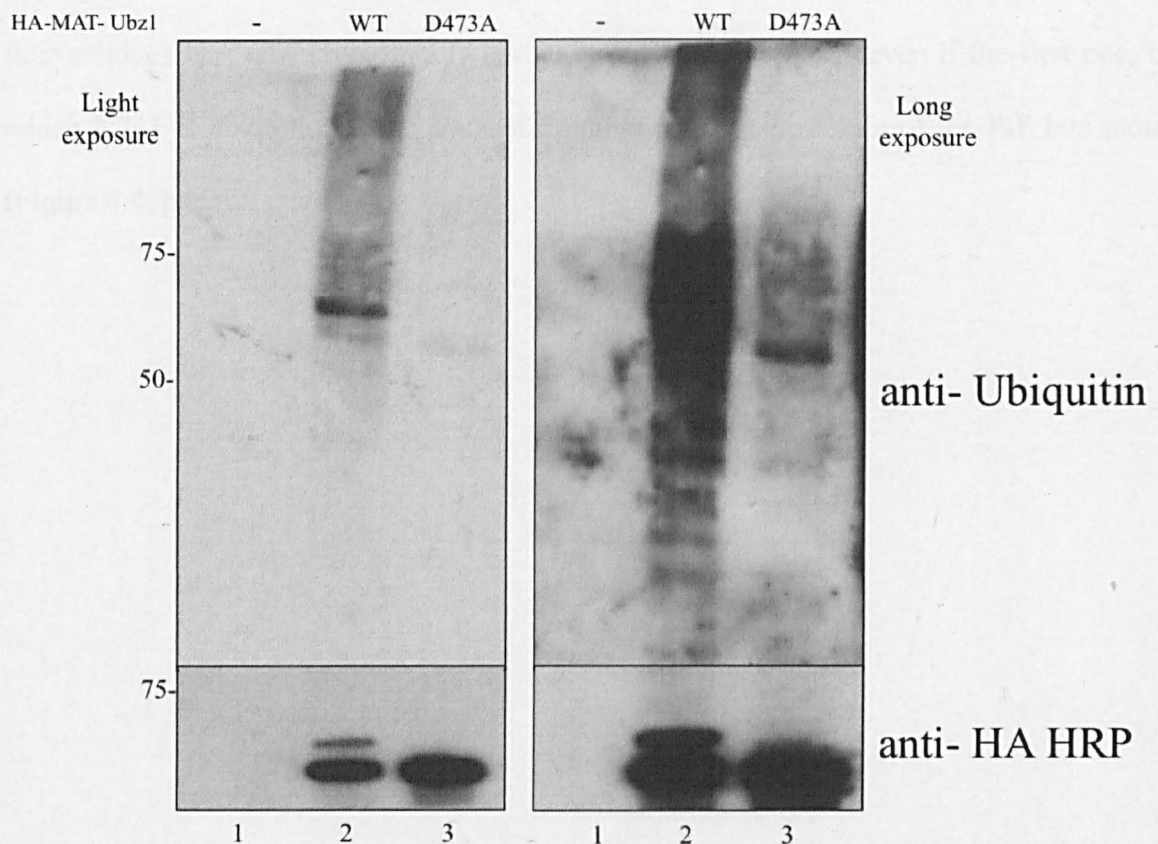
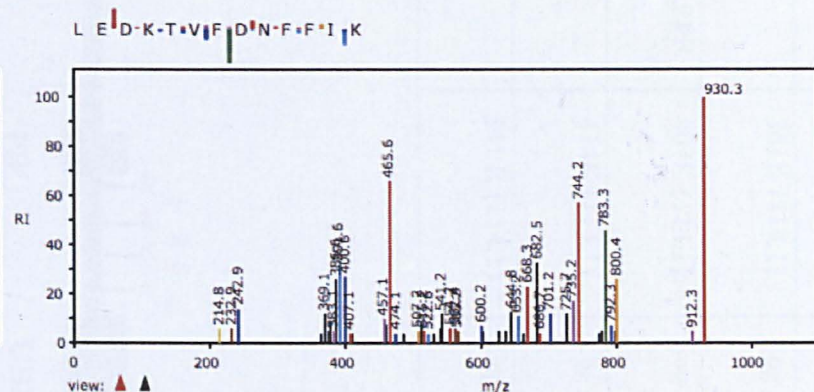
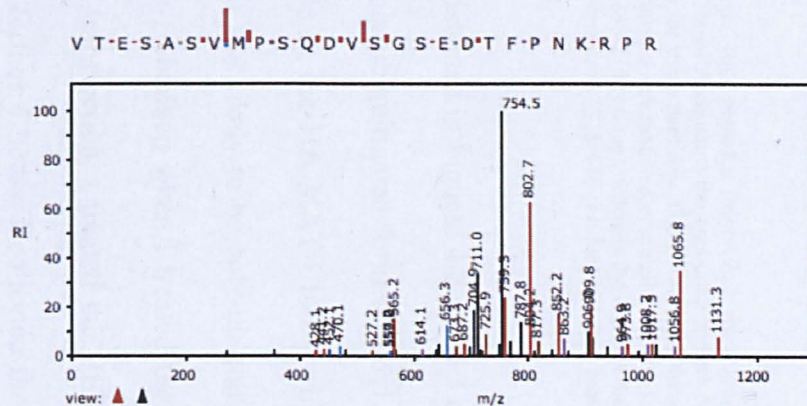
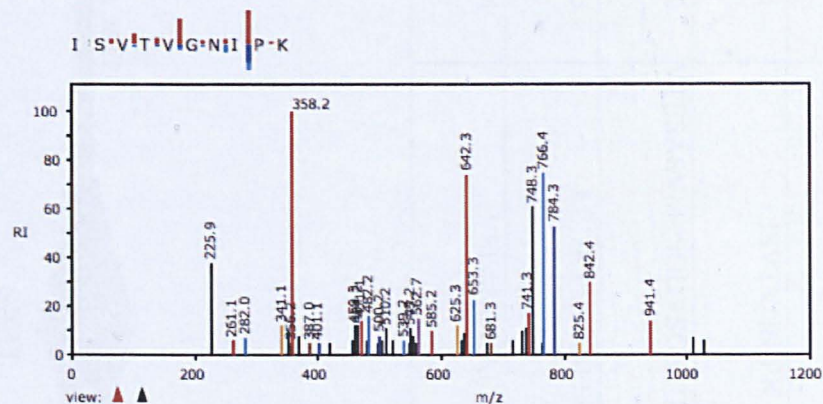
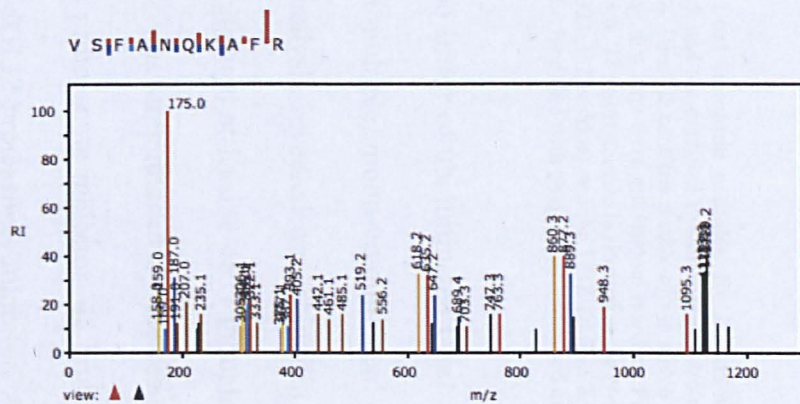


Figure 4.3. Ubz1 WT ubiquitination. HA-MAT-Ubz1 WT and D473A were expressed in HEK293T cells, purified and eluted with the peptide. Samples were blotted with the anti-ubiquitin antibody and with anti-HA HRP antibody.

Samples analysed by LC-MS/MS also revealed the sites in Ubz1 that are ubiquitinated. The three C-terminal residues of the ubiquitin monomer are Arginine-Glycine-Glycine and the last C-terminal Glycine is conjugated to the Lysine of the protein target. Trypsin cleaves after Lysine and Arginine residues, but when Lysine residues are modified with ubiquitin, the tryptic cleavage leaves a di-Glycine tag, which shifts the mass of the peptide by 114.1 Da. Thus, peptides modified by ubiquitin can be identified both by

the increase in the mass and often by a missed cleavage at the ubiquitinated Lysine.

Analysing the samples by LC-MS/MS revealed four Ubz1 peptides that were ubiquitinated and the annotated spectra of their modified forms are reported in Figure 4.4 (panel A) with their specific sites that are listed in Figure 4.4 (panel B) and are represented by a cartoon in Figure 4.4 (panel C). Interestingly, I noticed that all of the modified Lysines were located between the PIP box motif and the UBZ domain, but none of these four residues was positioned in any particular annotated domain, even if the first one, in which K341 is ubiquitinated, is situated 9 amino acids downstream of the PIP box motif (Figure 4.4, panel B and C).



B

PEPTIDE	SEQUENCE MODIFIED	MODIF.	E-VALUE
352-361	ISVTVGNIPK	361K (114.1)	Log ₁₀ (-4.8)
385-410	VTESASVMPSQDVSGSEDTFPNKRPR	407K (114.1)	Log ₁₀ (-3.3)
411-423	LEDKTVFDNFFIK	414K (114.1)	Log ₁₀ (-1.5)
335-344	VSFANQKAFR	341 K (114.1)	Log ₁₀ (-4.8)

C

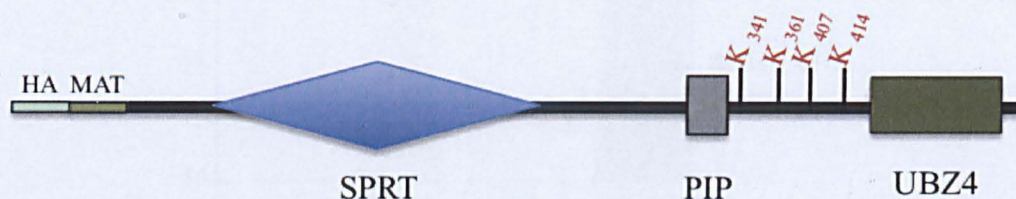


Figure 4.4. Ubz1 ubiquitin peptides. By LC-MS/MS analysis, four peptides from Ubz1 were found to carry the ubiquitin mark and the modified Lysine residues are reported. Panel A contains the annotated spectra for the modified Ubz1 peptides. Panel B contains a table of the match statistics for these peptides. The E-values of these peptides were low, indicating that they were not random matches. Panel C shows a schematic representation of Ubz1. Ubz1 contains 489 amino acids. The Sprt domain is situated at the N-terminus (45-213 residue), whereas the PIP box motif is closer the C-terminus (325-332 residue) and the UBZ domain is at the C-terminus (345-476 residue) of Ubz1 sequence. The four Lysine residues modified with ubiquitin are coloured in red.

The smear of ubiquitinated Ubz1 that I detected in Figures 4.2 and 4.3 corresponds to either a multiple, mono-ubiquitinated or a poly-ubiquitinated form of Ubz1. Since LC-MS/MS analysis revealed one ubiquitin peptide in the HA-MAT-Ubz1 purification that was ubiquitinated at Lysine 48, I thought that it was likely to be poly-ubiquitinated. This hypothesis was strengthened by the results that I obtained when I treated the transfected cells with proteasome inhibitor. 48 hours after transfection, I treated the HEK293T cells with the MG132 proteasome inhibitor. After a further 4 hours, I collected the cells and I

immunopurified HA-MAT-Ubz1 as described above. The HA peptide eluted samples were blotted with the anti-ubiquitin antibody and as shown in Figure 4.5, the level of ubiquitinated Ubz1 greatly increased upon treatment. This demonstrates that the ubiquitinated Ubz1 is degraded by the proteasome and further confirms the finding by LC-MS/MS that the Ubz1 carries poly-ubiquitin chains linked at K48. In the anti-HA HRP blot two bands for HA-MAT-Ubz1 are visible, which probably correspond to the unmodified and mono-ubiquitinated form of Ubz1 (Figure 4.5, bottom panel).

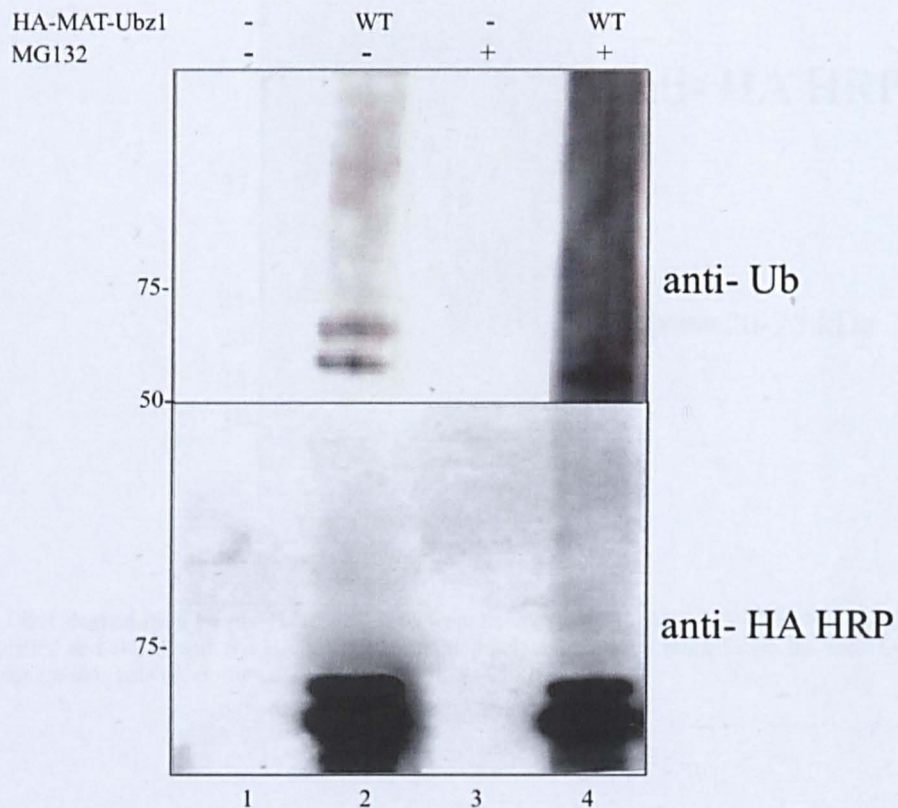


Figure 4.5. Ubz1 poly-ubiquitination. HA-MAT-Ubz1 WT was expressed in HEK293T cells. After 48 hours, cells were treated with MG132 for an additional 4 hours. Ubz1 was HA purified, eluted with the peptide and blotted with the anti-ubiquitin antibody. Anti-HA HRP antibody was used as a control.

4.1.2. Ubz1 Autocleavage

I also noticed that when analysed by SDS-PAGE and western blot, in addition to the 55 kDa full length HA-MAT-Ubz1, other immune-reactive bands were also present.

The upper band in the WT HA-MAT-Ubz1 most likely corresponds to the mono-ubiquitinated form of Ubz1, but there were abundant bands detected between 25 and 50 kDa, as shown in Figure 4.6.

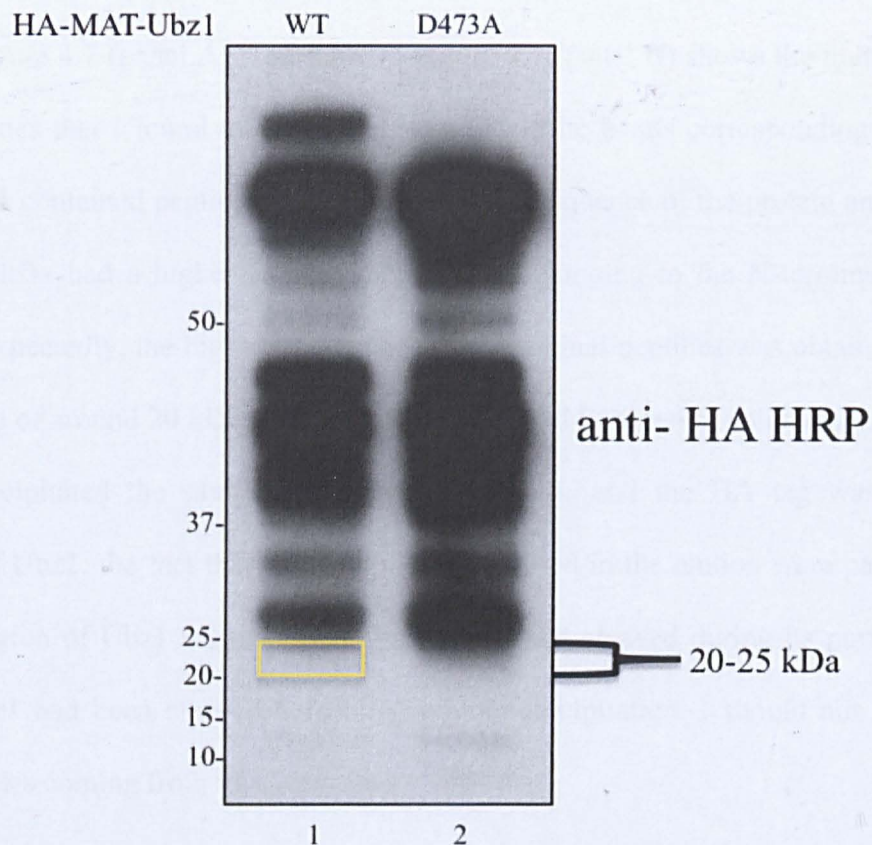
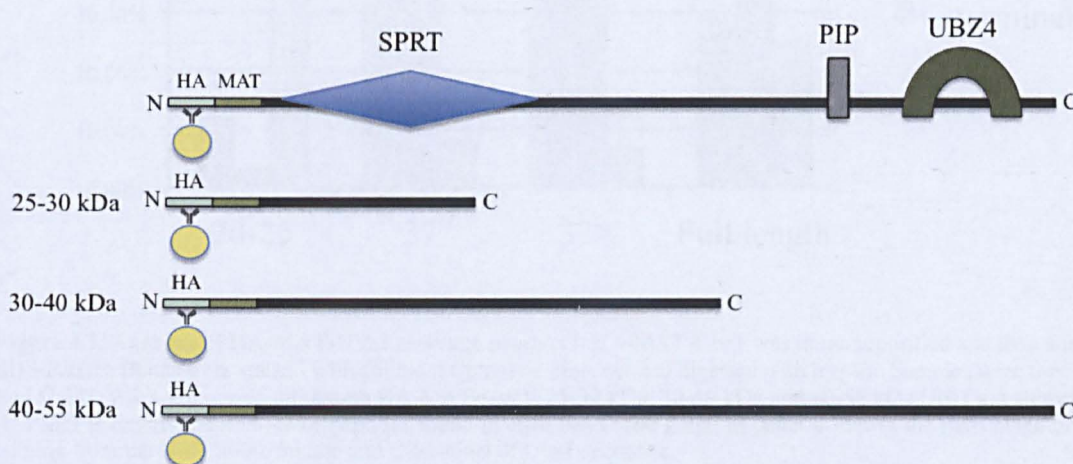


Figure 4.6. Ubz1 degradation bands. HEK293T cells were transfected with HA-MAT-Ubz1 WT and D473A. Samples were HA purified and eluted with the HA peptide. Immunoprecipitations were blotted with the anti-HA HRP antibody. The yellow square indicates the portion of the gel between 20-25 kDa.

I thought that the lower bands might correspond to degraded forms of Ubz1. To investigate this further, I immunoprecipitated HA-MAT-Ubz1 WT and I eluted the immune-complexes with the HA peptide. I ran the samples on a gel, and various regions of the gel were digested and analyzed by LC-MS/MS. Since the HA-MAT-Ubz1 constructs are N-terminally tagged and HA purified, the degradation products should also contain the N-terminus.

I divided the full-length sequence into N-terminal, middle and C-terminal bins and I determined the percentage of the peptides coming from each of the three bins. Assuming that the N-terminus was intact, the three regions would correspond to a band up to 30 kDa for the N-terminal region, the middle region up to 40 kDa and the C-terminal region up to 55 kDa, Figure 4.7 (panel A). The table in Figure 4.7 (panel B) shows the number of total Ubz1 peptides that I found in each band. I saw that the bands corresponding to the full-length Ubz1 contained peptides that cover the entire sequence of the protein and the bands around 37 kDa had a higher amount of peptides belonging to the N-terminal region of Ubz1. Unexpectedly, the highest percentage of C-terminal peptides was obtained from the lowest band of around 20 kDa (Figure 4.7, panel C and Figure 4.6, yellow square). Since I immunoprecipitated the samples with anti-HA beads, and the HA tag was at the N-terminus of Ubz1, the fact that some peptides identified in the elution were part of the C-terminal region of Ubz1 suggests that Ubz1 was being cleaved during its purification. In fact, if Ubz1 had been cleaved before its immunoprecipitation, I should not have found Ubz1 peptides coming from its C-terminus.

A



B

BAND'S SIZE	TOTAL NUMBER OF Ubz1 PEPTIDES
20-25 kDa	346
37 kDa	326
37> kDa	504
FULL LENGTH	977

C

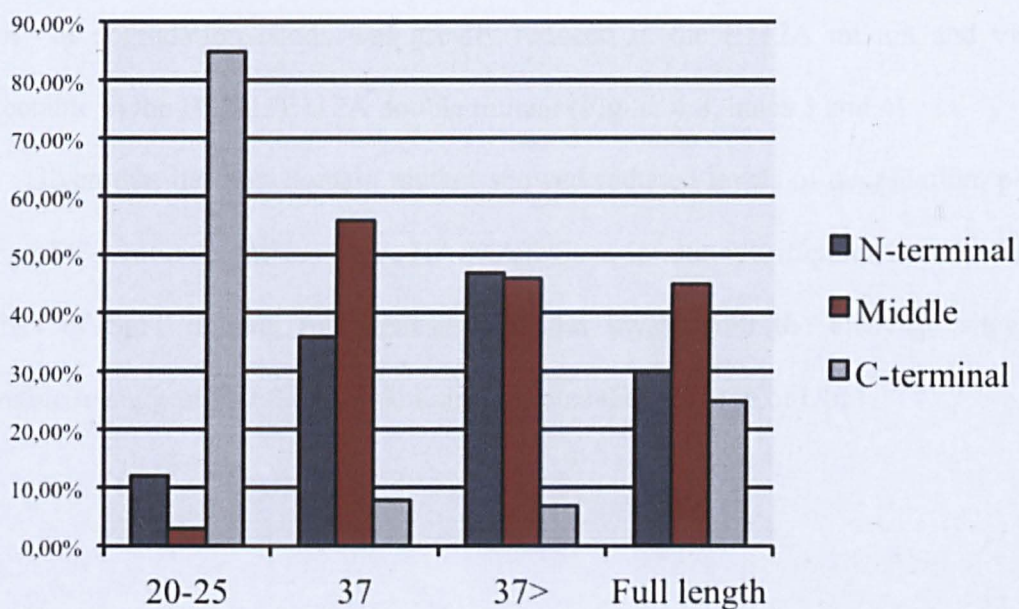


Figure 4.7. Analysis of HA-MAT-Ubz1 cleavage products. HA-MAT-Ubz1 was immunopurified and then analyzed by SDS-PAGE. Bands were stained with colloidal Coomassie blue, cut and digested with trypsin. Samples were then analysed by LC-MS/MS. Cartoons of full length HA-MAT-Ubz1, 25-30 kDa, 30-40 kDa and 40-55 kDa Ubz1 are shown in panel A. Panel B reports the number of peptides found in each band. The graph in panel C shows the percentage of peptides coming from the N-terminal, middle and C-terminal of Ubz1 sequence.

As previously noted, Ubz1 is the least characterized UBZ domain-containing

protein. In addition to the UBZ domain, Ubz1 also contains a PIP box motif and an Sprt domain. The Sprt domain of Ubz1 contains a HExH motif that is highly conserved from *E. coli* to humans and is similar to some zinc-dependent metalloprotease catalytic sites in which the zinc molecule is coordinated by two Histidines residues and a Glutamate residue. A water molecule acts as a nucleophile during catalysis (Jongeneel et al., 1989). Thus, I thought that the Sprt domain of Ubz1 might act as a metalloprotease, and mediates its own cleavage. To test this hypothesis, I analysed the behaviour of two Sprt domain mutants of Ubz1. I used site-direct mutagenesis to create two mutants of this domain; Glutamate at the position 112 of Ubz1, was substituted with an Alanine giving an Sprt domain single mutant (E112A), while in the Sprt double mutant, the Histidine 111 was substituted with Leucine residues in conjunction with the Glutamate substitution (H111L/E112A). When I analysed these Sprt mutants by western blot, I saw that the quantity of degradation bands was greatly reduced in the E112A mutant and virtually undetectable in the H111L/E112A double mutant (Figure 4.8, lanes 3 and 4).

Given that the Sprt domain mutant showed reduced levels of degradation, plus the finding of C-terminal peptides in the HA-MAT-Ubz1 immunopurification and the fact that the sites of SprT domain mutagenesis were far away from the cleavage sites, it is reasonable to suggest that the Sprt domain mediates the cleavage of Ubz1.

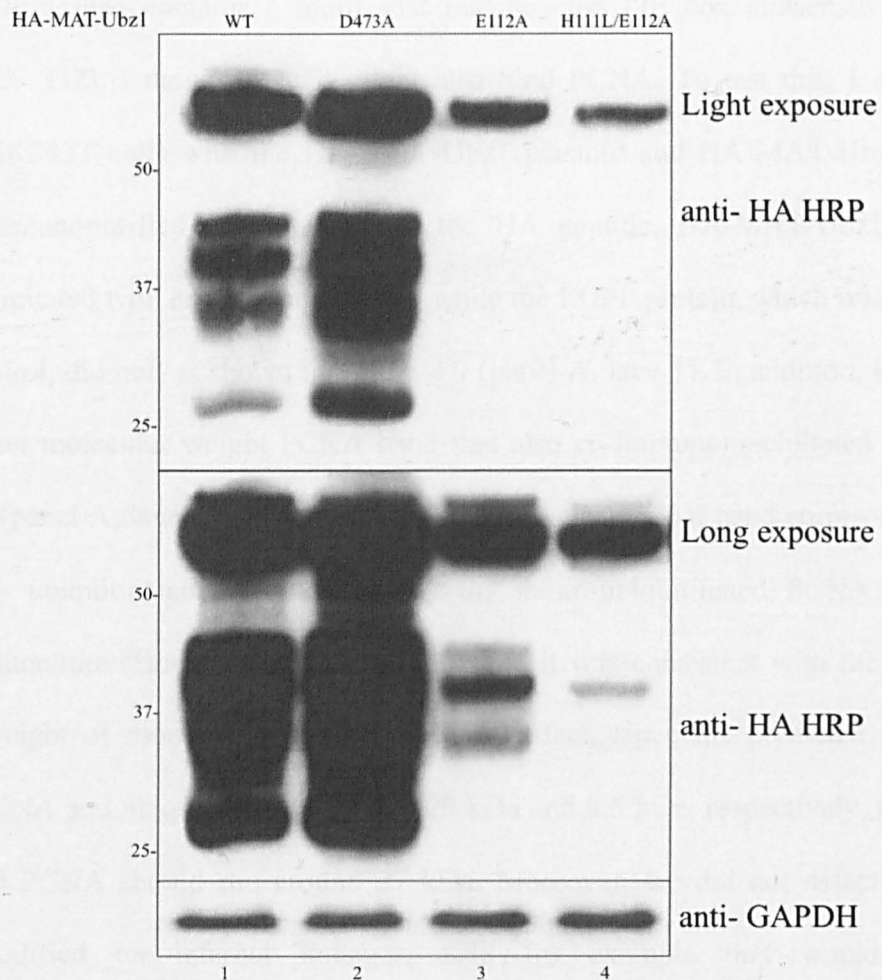


Figure 4.8. The HExH motif is required for Ubz1 degradation. HA-MAT-Ubz1 WT, D473A, E112A and H111L/E112A were expressed in HEK293T cells and lysates were blotted with the anti-HA HRP antibody.

4.2. Ubz1 Binds PCNA

4.2.1. Endogenous PCNA

PCNA plays an important role in DNA replication and repair. As a matter of fact, it acts as a DNA polymerase processivity factor, tethering DNA polymerases onto DNA strands and when the replication fork stalls upon DNA damage, it can be mono or poly-ubiquitinated.

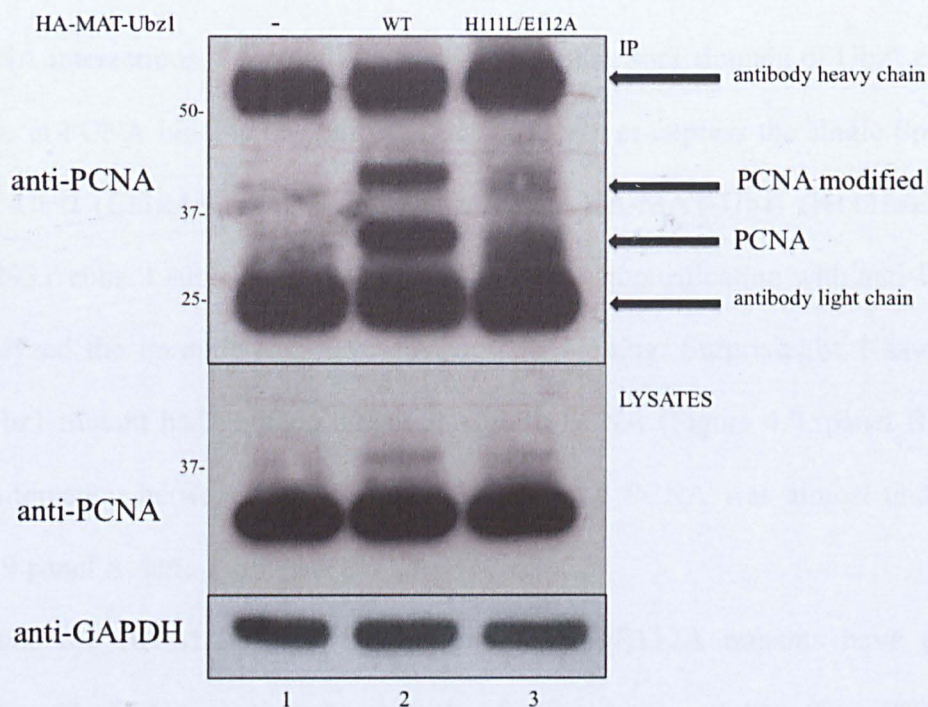
Many proteins possess a PCNA interacting motif (PIP box), which mediates their interaction with PCNA. Three UBZ containing proteins possess a PIP box: polymerase η (Haracska et al., 2001), polymerase κ (Haracska et al., 2002) and SNM1A (Yang et al.,

2010). As Ubz1 also contains a motif that matches the PIP box consensus sequence (residues 325- 332), I thought that it might also bind PCNA. To test this, I decided to transfect HEK293T cells with the HA-MAT-Ubz1 plasmid and HA-MAT-Ubz1 protein was then immunopurified and eluted with the HA peptide. HA-MAT-Ubz1 WT co-immunoprecipitated with endogenous PCNA, while the EGFP protein, which was used as a negative control, did not, as shown in Figure 4.9 (panel A, lane 1). In addition, I noticed a second, higher molecular weight PCNA band that also co-immunoprecipitated with HA-MAT-Ubz1 (panel A, lane 2, panel B, lane 1). It is not clear if that band correspond to the mono or di- ubiquitinated PCNA, but only the mono-ubiquitinated PCNA has been reported in literature (Hoege et al., 2002), I thought it was consistent with the predicted molecular weight of mono-ubiquitinated PCNA. In fact, since the predicted molecular weight of PCNA and ubiquitin monomer are 29 kDa and 8.5 kDa, respectively, the mono-ubiquitinated PCNA should run around 37 kDa. Moreover, we did not detect ubiquitin peptides modified on internal linkages, K48 for example that would indicate diubiquitination.

Since I already found that Ubz1 is subjected to coupled ubiquitination, which also happens to WHIP and Pol η , whose UBZ domains binds ubiquitin, I thought that the UBZ domain of Ubz1 might also bind ubiquitin. Therefore, in order to test if this domain might contribute to the interaction between Ubz1 and the modified form of PCNA, I decided to check whether the UBZ mutant retains the capacity to bind the modified form of PCNA. Therefore, I over-expressed the HA-MAT-Ubz1 D473A mutant in HEK293T cells and I purified Ubz1 from the whole cell extracts. When I blotted the HA purified immune-complexes, I saw that the UBZ domain mutants retained the capacity to bind the unmodified but not the modified forms of PCNA, which I predicted to be mono-ubiquitinated PCNA, strengthening the hypothesis that this modification corresponds to ubiquitin. Nevertheless, when I immunopurified the UBZ domain mutant of Ubz1, a modified form of PCNA with a molecular weight very close to the unmodified form of

PCNA was detected, as shown in Figure 4.9 (panel B, lane 2), but the identity of this band is unknown. Moreover, as is visible in Figure 4.9 (panel B, lane 2, bottom panel), the UBZ domain mutant of HA-MAT-Ubz1 presents as only one band, which corresponds to the unmodified form of Ubz1, whereas the WT and the other mutants of Ubz1 showed a band of unmodified Ubz1 and an ubiquitinated form of Ubz1. These data are consistent with recent published results, in which it has been demonstrated that Ubz1 has a functional UBZ domain that directly binds ubiquitin (Centore et al., 2012).

A



B

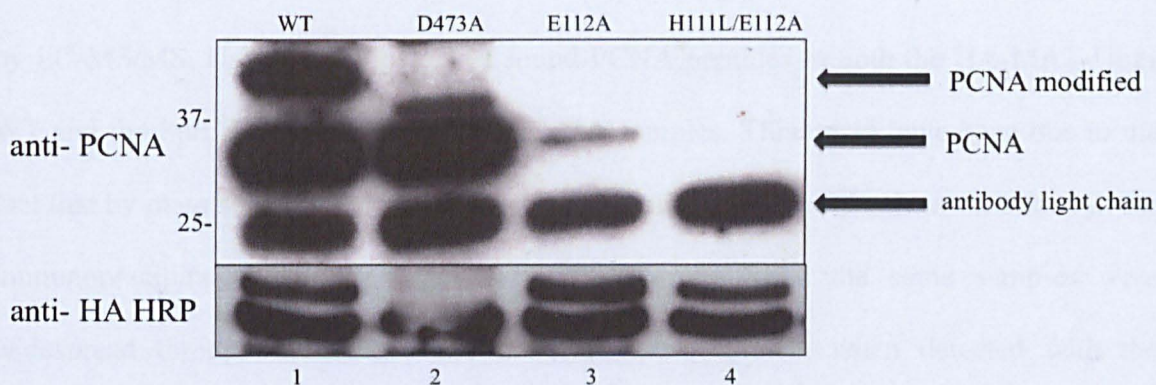


Figure 4.9. Ubz1 co-immunoprecipitates with PCNA. HA-MAT-Ubz1 WT, D473A, E112A or H111L/E112A were expressed in the HEK293T cells and HA-purified. In panel A are shown the IPs of the negative control (EGFP), WT and H111L/E112A blotted with the anti-PCNA antibody (high panel). Whole cell lysates were blotted with the anti-PCNA antibody as a control (lower panel). In panel B are shown IPs of WT, D473A, E112A and H111L/E112A blotted first with anti-PCNA antibody and then with anti-HA HRP antibody.

It has recently been reported that the PIP box motif mediates the interaction between Ubz1 and PCNA (Centore et al., 2012; Davis et al., 2012; Ghosal et al., 2012; Juhasz et al., 2012; Machida et al., 2012; Mosbech et al., 2012). In addition, my data demonstrate that Ubz1 interacts with PCNA and that the UBZ domain is indispensable for

the interaction with the ubiquitinated form of PCNA.

Given that both the PIP box motif and the UBZ domain are important for Ubz1/PCNA interactions, I decided to verify whether the SprT domain of Ubz1 could also play a role in PCNA binding. Therefore, I decided to over-express the single Sprt mutant HA-MAT-Ubz1 (E112A) and the double Sprt mutant HA-MAT-Ubz1 (H111L/E112A) in the HEK293T cells. I subjected the lysates to the immunopurification with anti-HA beads and I analyzed the immune-complexes by western blotting. Surprisingly, I saw that the E112A Ubz1 mutant had reduced interactions with PCNA (Figure 4.9, panel B, lane 3), and the interaction between H111L/E112A mutant and PCNA was almost undetectable (Figure 4.9 panel A, lane 3 and panel B lane 4).

Since the HA-MAT-Ubz1 E112A and H111L/E112A mutants have a weaker interaction with PCNA, I thought that the SprT domain might affect PCNA/Ubz1 interaction. To investigate this further, I digested a portion of HA purified HA-MAT-Ubz1 WT and the Sprt double mutant (H111L/E112A) with trypsin and analysed those samples by LC-MS/MS. Using this approach, I found PCNA peptides in both the HA-MAT-Ubz1 WT and the Sprt double mutant H111L/E112A samples. This could have been due to the fact that by mass spectrometry we analysed the entire quantity of peptides contained in the immunoprecipitated samples, whereas in the western blots the same samples were widespread throughout the gel and therefore more diluted, when detected with the antibody. However, I only found evidence of ubiquitination of PCNA in the WT samples (Table 4.2). In particular, I saw that PCNA was ubiquitinated at K164.

Ubz1 TYPE	UNIQUE PCNA	TOTAL PCNA	UBIQUITINATED PCNA
WT	6	19	K164
H111L/E112A	2	14	0

Table 4.2. Ubiquitinated PCNA from HA-MAT-Ubz1 WT and H111L/E112A purifications. HA-MAT-Ubz1 WT and H111L/E112A were purified with the HA beads. Immune-complexes were then digested with trypsin and peptides were analysed by LC-MS/MS. Here the total and unique peptides for PCNA found in the analysis are reported. The site of PCNA ubiquitination is reported in the forth column.

Even though I was not able to demonstrate that the higher band of PCNA is ubiquitinated by western blot, the mass spectrometry results are consistent with the higher molecular band of modified PCNA being mono-ubiquitinated form of PCNA.

K164 of PCNA is the residue where RAD6/RAD18 ubiquitinate PCNA following UV damage. However, it has been reported that a fraction of PCNA appears to be constitutively ubiquitinated at low levels in some cell lines, including the HEK293T cells, even in absence of DNA damage (Chiu et al., 2006). Since my data indicate that Ubz1 binds both the modified and the unmodified form of PCNA, I thought it would be advantageous to find a way of mimicking the situation in which PCNA was permanently ubiquitinated. Therefore, I decided to build three different plasmids that I will discuss in the next section.

4.3. PCNA Fusion Proteins

4.3.1. Construction of PCNA Fusion Proteins

Endogenous metabolic processes and environmental factors continuously damage genomic DNA. If the replication fork comes across a DNA lesion, the high fidelity polymerases stall because their active sites are not able to accommodate damaged bases.

Fork stalling can result in the mono-ubiquitination of PCNA at K164, which recruits the low fidelity UBZ domain containing DNA polymerases (Bienko et al., 2005; Hoege et al., 2002).

In order to reproduce the situation in which PCNA is mono-ubiquitinated, I generated a plasmid expressing HA-MAT tagged PCNA fused to mono-ubiquitin at the N-terminus (HA-MAT-Ub-PCNA) (Figure 4.10). Ramasubramanyan et al., performed different experiments in *S. pombe*, in which they fused a single ubiquitin molecule to the N-terminal end of PCNA. They demonstrated that the fusion protein was expressed at similar levels to the endogenous PCNA and that it mimicked the effects of mono-ubiquitinated PCNA. In particular, the Ub-PCNA fusion protein was still able to form a trimeric complex with the endogenous PCNA; moreover, yeast in which the K164 of endogenous PCNA was mutated, were still able to survive upon UV-irradiation when they were transformed with the Ub-PCNA construct (Ramasubramanyan et al., 2010). Other experiments were performed in *S. cerevisiae*, that revealed that the position of ubiquitin on PCNA was irrelevant for the interaction with the polymerase η (Parker et al., 2007).

Similarly, in order to reproduce the conditions where PCNA is poly-ubiquitinated, I cloned a tetra-ubiquitin fusion to PCNA (Figure 4.10). Ub-PCNA constructs were synthesized chemically by Genescript and the cDNAs were inserted into the pCGN-MAT vector. In pHA-MAT-Ub-PCNA, the N-terminal Methionine of PCNA was deleted in order to avoid the ubiquitin-specific proteases that would otherwise cleave the fusion protein, whereas in pHA-MAT-4Ub-PCNA, the N-terminal Methionine of the ubiquitins were substituted with Prolines and the N-terminal Methionine of PCNA was deleted.

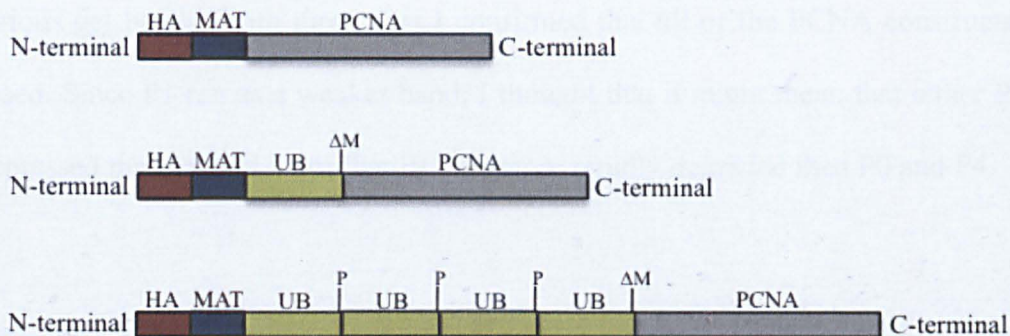


Figure 4.10. HA-MAT-PCNA fusion proteins. Cartoon of the PCNA fusion proteins. PCNA, Ub-PCNA and 4Ub-PCNA were cloned into pCGN-MAT resulting in epitope tagged versions. In order to suppress the cellular cleavage of the ubiquitin moiety the N-terminal Methionine of PCNA was deleted, whereas in HA-MAT-4Ub-PCNA the N-terminal Methionines between the four ubiquitins were also mutated to Prolines.

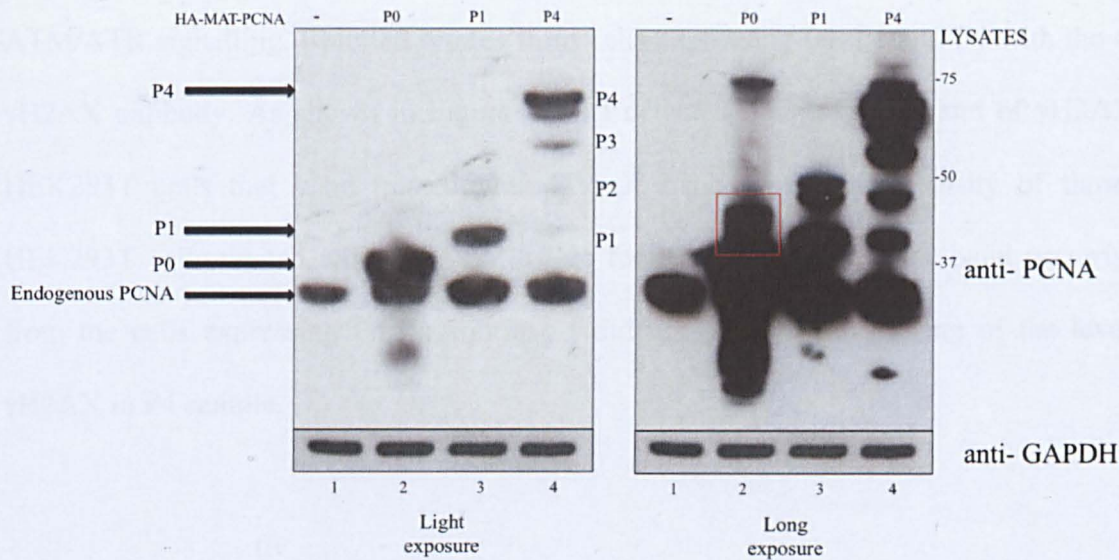
In this thesis, I will call the three constructs that I built, P0 (HA-MAT-PCNA), P1 (HA-MAT-Ub-PCNA) and P4 (HA-MAT-4Ub-PCNA).

4.3.2. Verification of PCNA Constructs

Once I obtained the three PCNA constructs, I decided to verify that the three PCNA fusion proteins were expressed. To do this, I transiently transfected 1 μ g of the three PCNA plasmids into the HEK293T cells and I analysed the whole cell lysates by western blotting with anti-PCNA antibody. I saw that all the fusion proteins were expressed, as reported in Figure 4.11 (panel A). In particular, P0 was approximately the same size as endogenous PCNA and P4 showed four bands, which probably correspond to the mono- di- or tri-ubiquitinated PCNA, labelled as P1, P2 and P3 (Figure 4.11, panel A, lane 4). All the forms of PCNA were easily detected when immunoprecipitations were blotted with the anti-HA HRP antibody, and if P1 ran as a weaker band, as shown in Figure 4.11 (panel B, lane 3). Moreover, P1 showed an additional band that seemed to have the appropriate size of HA-MAT-Ub (Figure 4.11, panel B, lane 3), whereas P4 showed four additional bands, which might correspond to the HA-MAT-Ub, HA-MAT-di-Ub, HA-MAT-tri-Ub and HA-MAT-tetra-Ub (Figure 4.11, panel B, lane 4, indicated with asterisk), but in order to verify that these bands are HA-MAT-ubiquitin isoforms, I would need to perform LC-MS/MS on

the various gel bands. With these data I confirmed that all of the PCNA constructs were expressed. Since P1 ran as a weaker band, I thought that it might mean that either P1 was less expressed than P0 and P4 or that P1 was more rapidly degraded then P0 and P4.

A



B

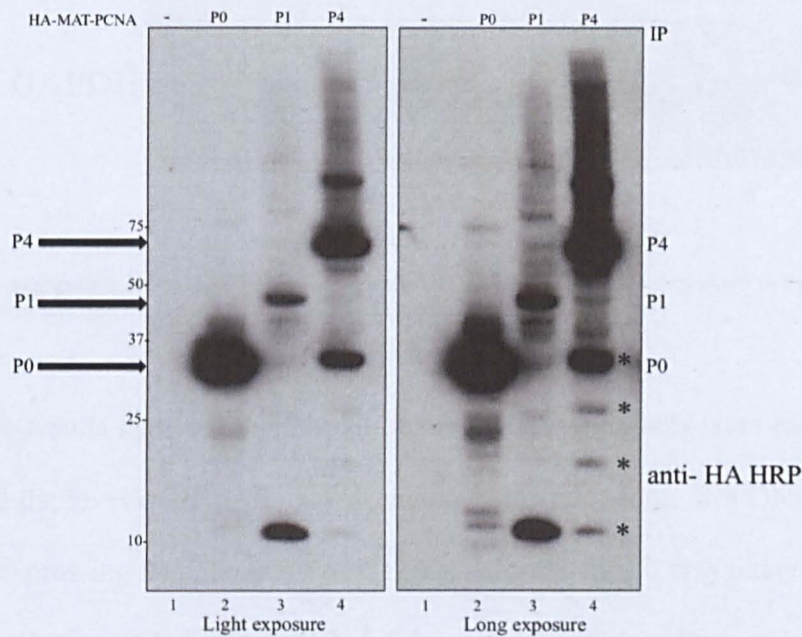


Figure 4.11. Verification of PCNA constructs. P0, P1 and P4 were expressed in the HEK293T cells and HA purified. In panel A the lysates of P0, P1 and P4 blotted with the anti-PCNA antibody are shown. In panel B an immunoprecipitation of P0, P1 and P4 blotted with the anti-HA HRP antibody is shown.

Given that PCNA is mono-ubiquitinated upon DNA damage, I decided to test if expression of P1 could be a signal of DNA damage. As I described in paragraph 1.1, upon DSBs, H2AX is phosphorylated by the kinases ATM and ATR. Since the phosphorylated H2AX is a sensitive target for DSBs in cells, I decided to use the γ H2AX antibody to check if expression of the three PCNA constructs would result in increasing levels of ATM/ATR signalling. I blotted lysates from cells expressing P0, P1 and P4 with the anti- γ H2AX antibody. As shown in Figure 4.12, I detected the strongest band of γ H2AX in HEK293T cells that were treated with UV. A band of similar intensity of those of HEK293T cells treated with UV was visible for P1 and a less intense band was visible from the cells expressing P0. In contrast, I did not detect an increasing of the level of γ H2AX in P4 sample.

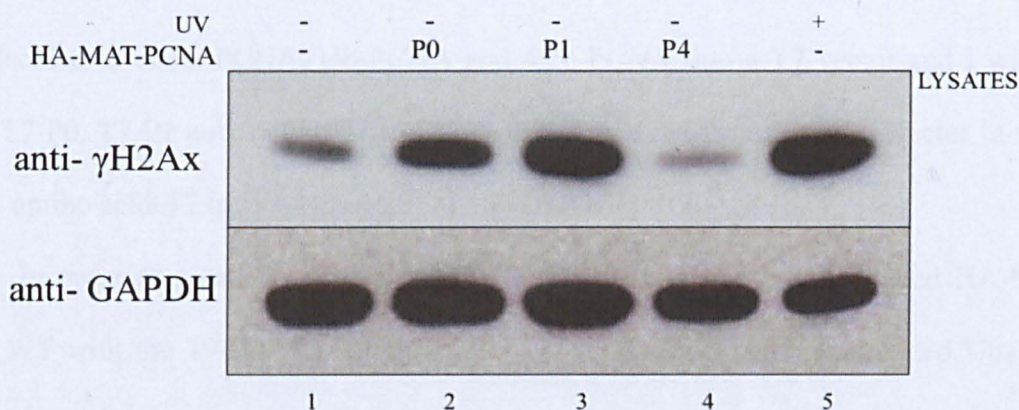


Figure 4.12. P1 expression activates a DNA damage signal. P0, P1 and P4 were expressed in the HEK293T cells and lysates were blotted with anti- γ H2AX and anti-GAPDH (loading control).

These results demonstrate that all of the PCNA constructs were expressed and that P1 increased the level of γ H2AX, which indicates the activation of a DNA damage signal in the cells expressing P1. The band of γ H2AX detected for P0 was likely the result of the mono-ubiquitination of HA-MAT-PCNA (Figure 4.11, panel A, long exposure, lane 2, red square), which could serve to increase the level of the damage signal.

4.4. Ubz1 and Ub-PCNA Co-expression

RAD6/RAD18 act together in ubiquitinating PCNA at K164 in response to DNA damage (Hoege et al., 2002) and recently the RAD18-dependent PCNA mono-ubiquitination has been reported to be indispensable for Ubz1 localization at damage foci (Centore, Yazinski et al. 2012). In fact, it has been shown that Ubz1 is recruited to the sites of damage in a manner dependent on both the PIP box motif and the UBZ domain (Machida, Kim et al. 2012; Centore, Yazinski et al. 2012).

In the first part of my results, I demonstrated that endogenous PCNA co-immunoprecipitates with HA-MAT-Ubz1. To further characterize the Ubz1/PCNA interaction, I decided to make use of the P0, P1 and P4 constructs as a model to reproduce the situation in which PCNA is unmodified, mono-ubiquitinated or poly-ubiquitinated. In the previous experiments I utilized Ubz1 and PCNA plasmids with the same HA-MAT tag, so I decided to clone PCNA, Ub-PCNA and 4Ub-PCNA into a T7 vector and I will call them T7-P0, T7-P1 and T7-P4. The pCGT7 vector derived from the pCG vector in which the 11 amino acid-T7 tag was inserted (MASMTGGQMG).

In order to characterize the Ubz1/PCNA interactions, I co-transfected HA-MAT-Ubz1 WT with the T7-P0, T7-P1 and T7-P4 constructs. I immunoprecipitated Ubz1 and then I blotted the immune-complexes with the anti-T7 antibody. As shown in Figure 4.13, I observed that T7-P0 and T7-P4 were easily visible and that those bands had a strong intensity, whereas T7-P1 band was not detectable at all.

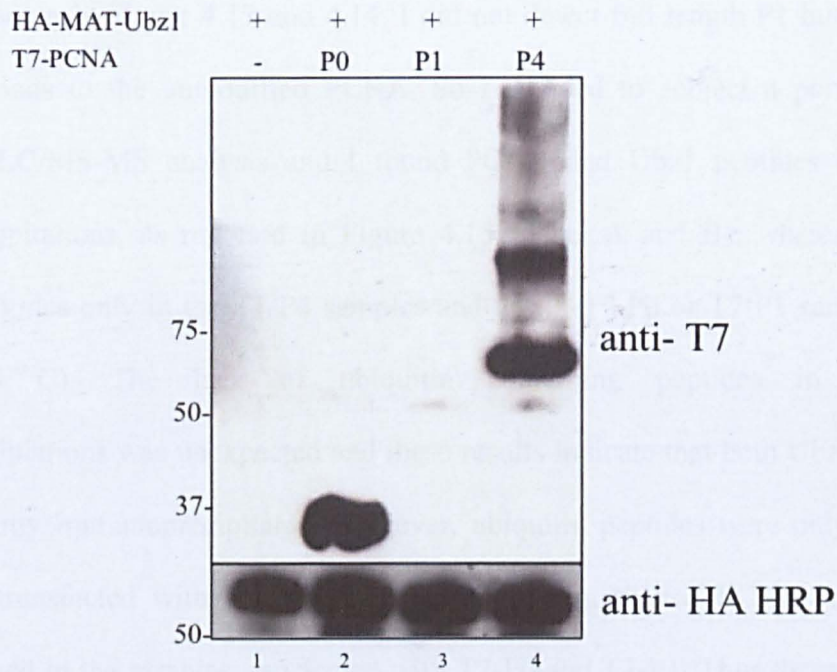


Figure 4.13. Co-expression of T7-P1 and Ubz1 results in a loss in T7-P1 immunoreactivity. T7-PCNA constructs were co-expressed with HA-MAT-Ubz1 in HEK293T cells. Lysates were HA purified and blotted with the anti-T7 and anti-HA HRP antibodies.

To investigate the fate of T7-P1, I decided to blot the immunopurified samples with an antibody against PCNA (Figure 4.14) and I saw that this antibody recognized bands of the appropriate size for T7-P0 and T7-P4 and a band very close to the molecular weight of T7-P0 in the immunoprecipitation from the cells transfected with T7-P1.

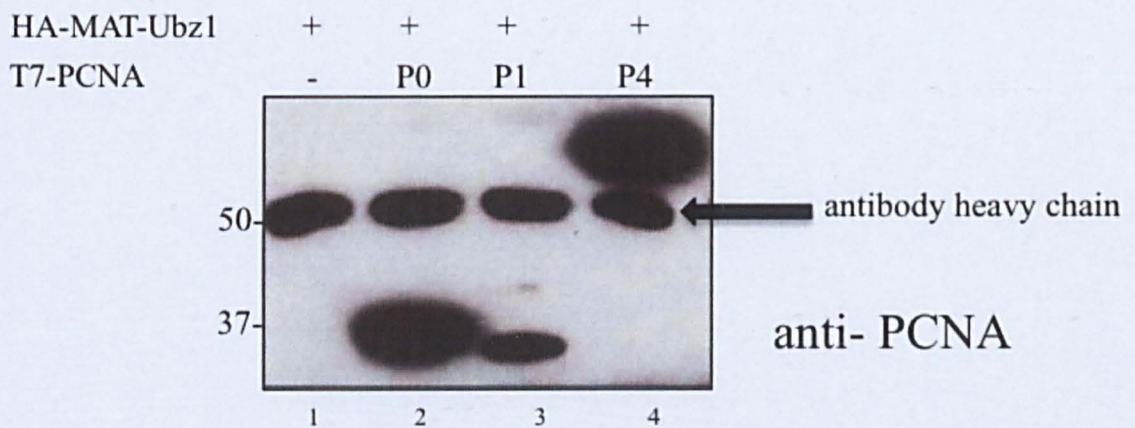
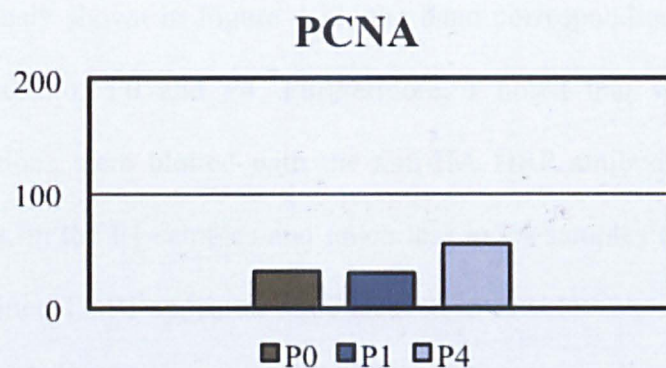


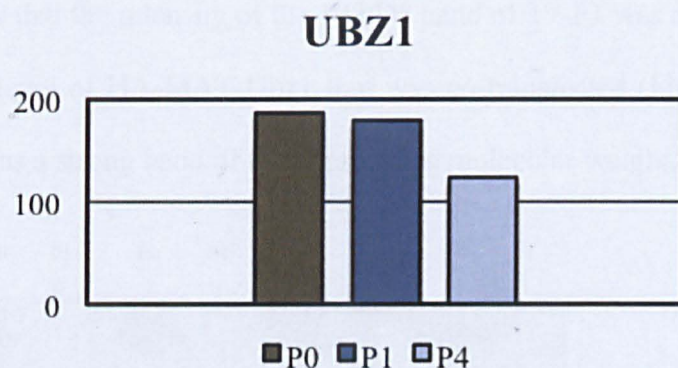
Figure 4.14. Ubz1 co-purifies with a fragment of T7-P1. T7-PCNA constructs were co-expressed with HA-MAT-Ubz1 in the HEK293T cells. Lysates were HA purified and immunoprecipitations were blotted with the anti-PCNA antibody.

As shown in Figure 4.13 and 4.14, I did not detect full length P1 but only a form that corresponds to the unmodified PCNA. So I decided to subject a portion of these samples to LC/MS-MS analysis and I found PCNA and Ubz1 peptides in all of the immunoprecipitations, as reported in Figure 4.15 (panel A and B), whereas I detected ubiquitin peptides only in the T7-P4 samples and not in T7-P0 or T7-P1 samples (Figure 4.15, panel C). The lack of ubiquitin containing peptides in the T7-P1 immunoprecipitations was unexpected and these results indicate that both Ubz1 and PCNA were efficiently immunoprecipitated. However, ubiquitin peptides were only detected in the sample transfected with T7-P4, indicating that ubiquitinated PCNA was absent or greatly reduced in the samples transfected with T7-P0 and T7-P1. Thus these data seemed to further support the results previously obtained from the western blot analysis.

A



B



C

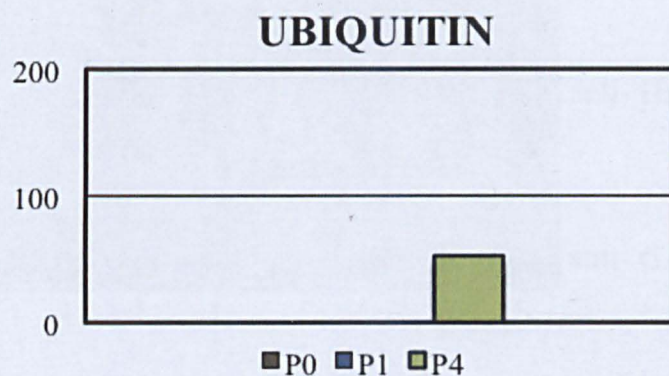


Figure 4.15. LC-MS/MS analysis of HA-MAT-Ubz1 and T7-PCNA co-immunoprecipitations. T7-P0, T7-P1 and T7-P4 were co-transfected with HA-MAT-Ubz1 WT. Samples were immunoprecipitated with the anti-HA beads, tryptic digested and analysed by LC-MS/MS. In panel A the peptides for PCNA are reported, whereas panel B and C indicate the number of Ubz1 and ubiquitin peptides, respectively.

4.4.1. Ub-PCNA Expression

As previously shown in Figure 4.11, the band corresponding to P1 had a weaker intensity than those of P0 and P4. Furthermore, I noted that when P0, P1 and P4 immunoprecipitations were blotted with the anti-HA HRP antibody, there was a lower band at ~10 kDa, in the P1 samples and much less in P4 samples (Figure 4.11, panel B, lanes 3 and 4). Since T7-P1 appeared to be cleaved in the blots shown in Figure 4.13 and 4.14, I decided to check if increasing levels of Ubz1 leads to an increasing intensity of 10 kDa band. Therefore, I co-transfected HEK293T cells with 1 μ g of T7-P0 or T7-P1 and 0, 1, 2, 3 μ g of HA-MAT-Ubz1. When I collected the lysates and I blotted them with an anti-T7 antibody, I saw that the intensity of the 10 kDa band of T7-P1 was directly proportional to the increasing level of HA-MAT-Ubz1 that was co-transfected (Figure 4.16), whereas T7-P0 always ran as a strong band of the appropriate molecular weight.

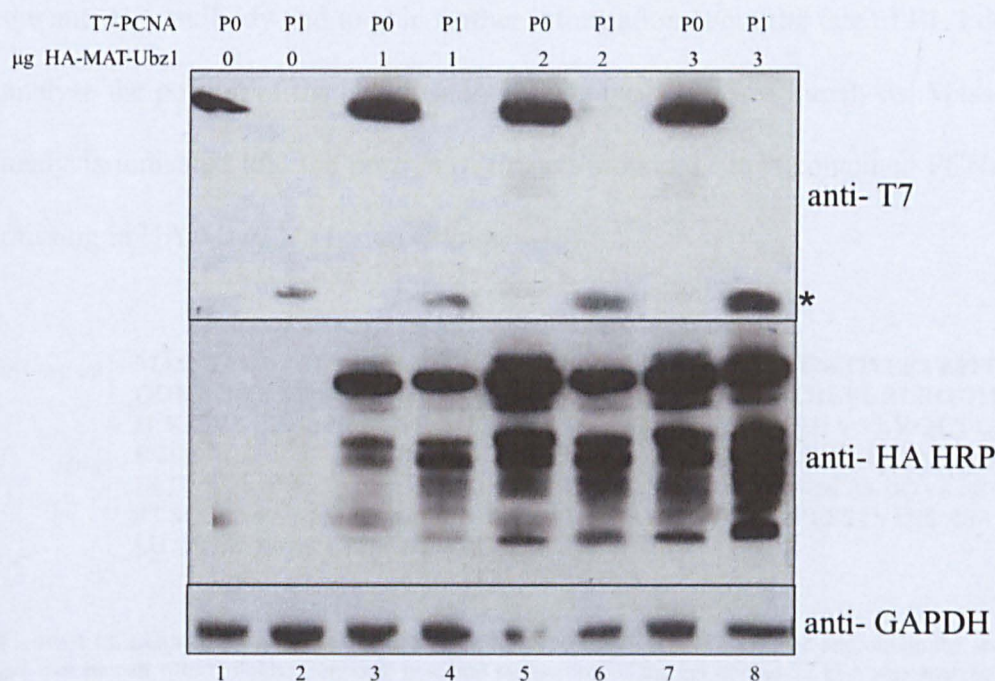


Figure 4.16. Tag-Ub-PCNA is cleaved to give rise to a 10 kDa band. T7-P0 and T7-P1 were co-transfected with 0, 1, 2, 3 μ g of HA-MAT-Ubz1. Lysates were blotted with the anti-T7 antibody and with the anti-HA HRP antibody as a control. Asterisk indicates the 10 kDa band.

In order to characterize the 10 kDa band, I ran the P1 immunopurifications on a gel and analyzed the bands by LC-MS/MS. This analysis revealed that the 10 kDa band in the P1 immunopurifications was HA-MAT tagged ubiquitin, in fact it ended very near to the junction between ubiquitin and PCNA, shown in Figure 4.17.

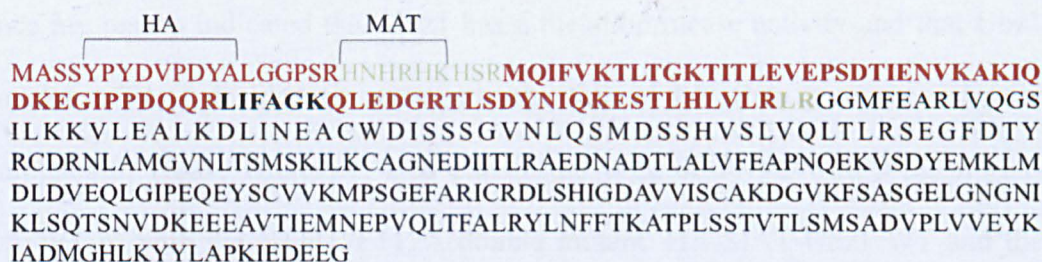


Figure 4.17. LC-MS/MS analysis of the 10 kDa band of P1. P1 was HA purified and run on the acrylamide gel. The gel was stained with colloidal Coomassie blue and the band around 10 kDa was analysed by LC-MS/MS. The resulting HA-MAT-Ub peptides are reported in red/green. The amino acid sequence corresponded to ubiquitin is shown in bold red.

Since the band corresponding to the other part of P1 would not be detectable with the anti-HA antibody and to gain further information about the fate of P1, I decided to also analyse the portion of the gel close to 37 kDa by LC-MS/MS analysis. Mass-spectrometry analysis indicated that the portion of the gel around 37 kDa contained PCNA, which was missing in HA-MAT-Ub fusion (Figure 4.18).

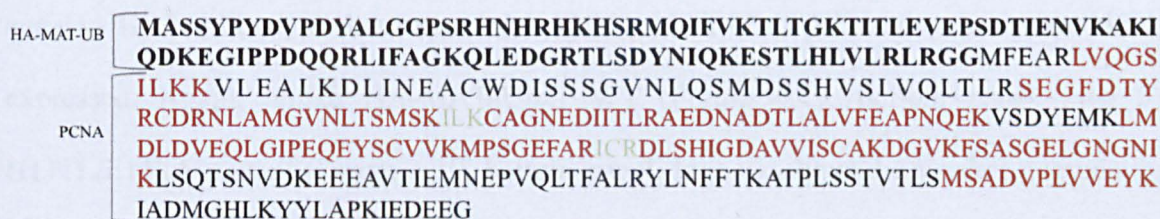


Figure 4.18. LC-MS/MS analysis of the 37 kDa band of P1. P1 was HA purified and run on the acrylamide gel. The gel was stained with colloidal Coomassie blue and the portion of the gel around 37 kDa was analyzed by LC-MS/MS. Amino acids identified by LC-MS/MS are shown in red and the amino acid sequence corresponded to HA-MAT-Ub is shown in black bold.

Considering the results from the mass spectrometry analysis, in which I only found HA-MAT-Ub peptides in the 10 kDa band and PCNA peptides in the 37 kDa band and the previous finding, in which a PCNA band at the same level of the P0 was found only when

P1, and not P4 was co-transfected with Ubz1, I believe that the band that I detected for T7-P1 in the anti-PCNA blot originates from P1 rather than from endogenous PCNA.

4.4.2. Ubz1 Mutants and Ub-PCNA Co-expression

Since my results indicated that Ubz1 has a metalloprotease activity and that Ubz1 appears to cleave P1, I decided to investigate the fate of P1 when co-expressed with different mutants of Ubz1. Therefore, I co-transfected 1 µg of T7-P1 with 9 µg of HA-MAT-Ubz1 WT or with the H111L/E112A double mutant. HA-MAT-Ubz1 WT and the double mutants were immunoprecipitated and eluted with the HA peptide. When I blotted these immune-complexes with the anti-T7 HRP antibody, as shown in Figure 4.19 (upper panel), I saw that the P1 appeared as a weak band when co-transfected with the HA-MAT-Ubz1 WT (Figure 4.19, upper panel, lane 5). This band did not correspond to the expected molecular weight of P1, which should be around 50 kDa, but may correspond to a modified form of P1. However, when T7-P1 was co-transfected with the H111L/E112A mutant, I noticed that P1 appeared as a smear of higher molecular weight forms (Figure 4.19, upper panel, lane 6) that I thought might be poly-ubiquitinated forms of PCNA. Expression of HA-MAT-Ubz1 was confirmed by blotting the same membrane with the anti-HA HRP antibody and as expected, both HA-MAT-Ubz1 WT and mutant were highly expressed. Again, unlike HA-MAT-Ubz1 WT (Figure 4.19, bottom panel, lane 5), H111L/E112A mutant (Figure 4.19, bottom panel, lane 6), showed a weaker degradation activity.

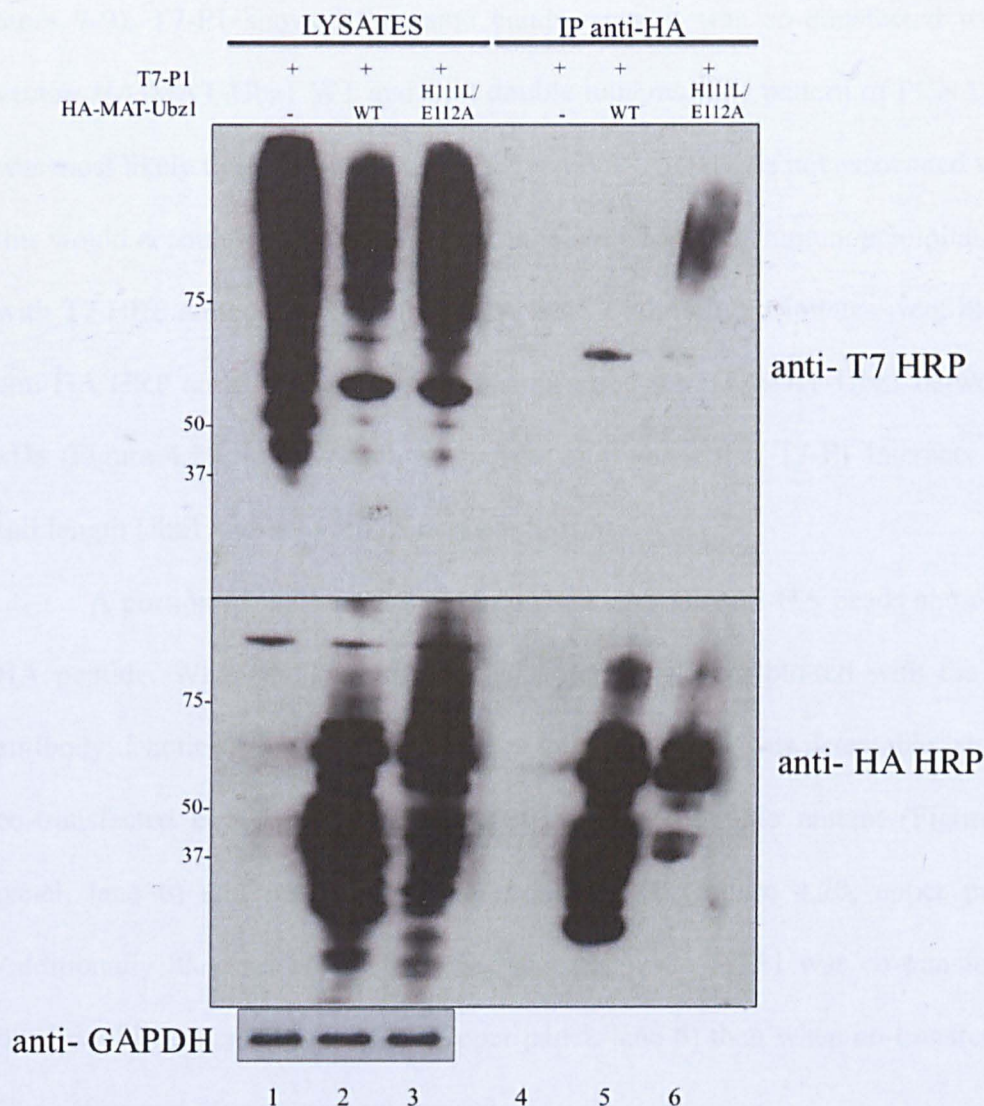


Figure 4.19. P1 smear. T7-P1 was co-transfected with HA-MAT-Ubz1 WT or H111L/E112A mutant. Samples were purified with HA beads and eluted with HA peptide. Immune-complexes were blotted first with the anti-T7 HRP antibody and then with anti-HA HRP antibody, as a control. Lysates blot is shown in lanes 1-3.

These results indicate that the T7-P1 appears to have a different fate when it was co-transfected with the Ubz1 SprT double mutant. In order to further investigate this, I performed the same co-transfection and I immunoprecipitated PCNA rather than Ubz1. I co-transfected 2 μ g of T7-PCNA with 9 μ g of HA-MAT-Ubz1 and samples were immunopurified with anti-T7 beads and blotted first with anti-T7 HRP antibody, stripped and then re-probed with anti-HA HRP antibody. As shown in Figure 4.20 (upper panel,

lanes 7-9), T7-P1 showed the same bands when it was co-transfected with the empty vector, HA-MAT-Ubz1 WT and SprT double mutants. The pattern of PCNA in the T7-IPs was most likely the result of a pool of tagged PCNA that was not associated with Ubz1 and this would account for the difference seen when Ubz1 was immunoprecipitated and probed with T7 HRP antibodies. Notably, when the T7 immunoprecipitates were blotted with the anti-HA HRP antibody, only two bands appeared for HA-MAT-Ubz1 between 50 and 75 kDa (Figure 4.20, lower panel, lanes 8-9), suggesting that T7-P1 interacts only with the full length Ubz1 and not with its degraded forms.

A portion of the samples were purified with the anti-HA beads and eluted with the HA peptide. When the immunopurified complexes were blotted with the anti-T7 HRP antibody, I noticed that the band corresponding to T7-P1 was detectable when T7-P1 was co-transfected both with the HA-MAT-Ubz1 SprT double mutant (Figure 4.20, upper panel, lane 6) and with the HA-MAT-Ubz1 WT (Figure 4.20, upper panel, lane 5). Additionally, the smear of T7-P1 was stronger when T7-P1 was co-transfected with the SprT double mutant (Figure 4.20, upper panel, lane 6) than when co-transfected with WT Ubz1 (Figure 4.20, upper panel, lane 5).

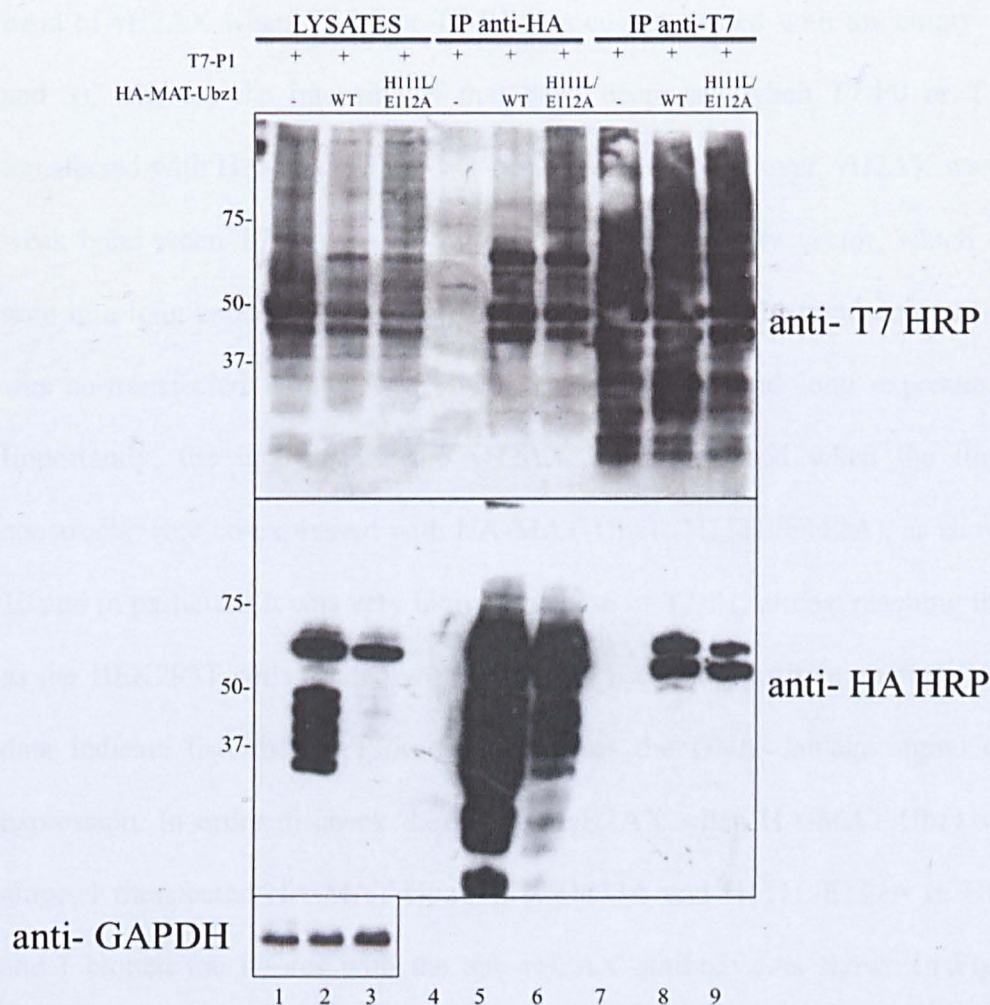


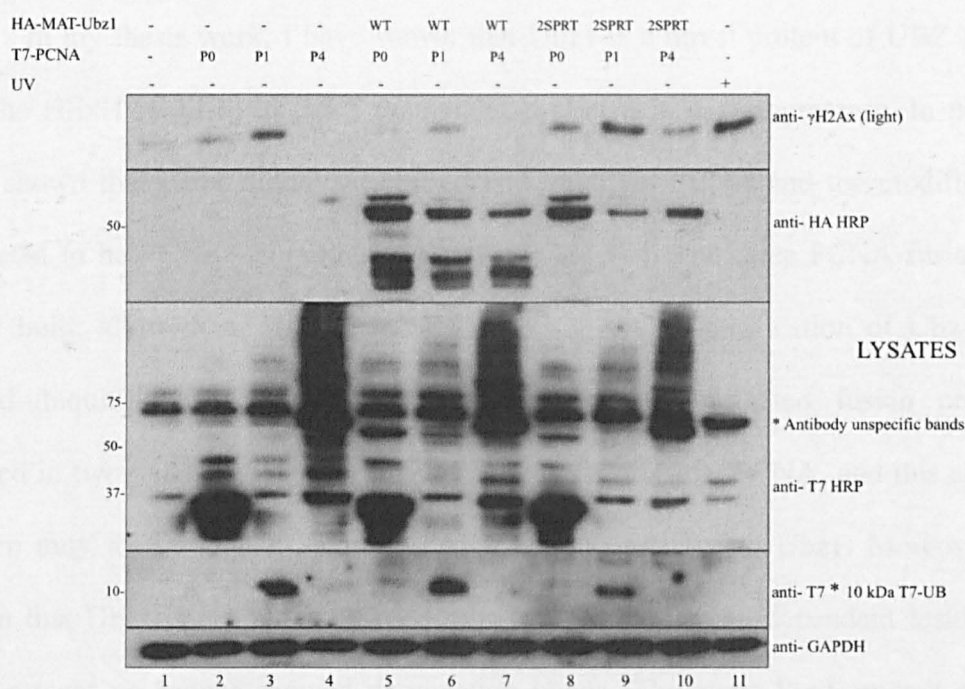
Figure 4.20. Change in T7-P1 level expression. 2 μ g of T7-P1 was co-transfected with 9 μ g of both HA-MAT-Ubz1 WT and H111L/E112A mutant. Samples were purified with the anti-T7 or anti-HA beads. Immune-complexes were blotted first with the anti-T7 HRP antibody and then with anti-HA HRP antibody. Lysates blot is shown in lanes 1-3.

4.5. Role of Ubz1 in DNA Damage

Given the results that showed P1 activated a DNA damage signal (Figure 4.12) and that the SprT domain of Ubz1 is playing a role in regulating PCNA ubiquitination, I decided to check if Ubz1 could also affect the level of the signal for DNA damage induced by P1 expression. Therefore, I co-transfected T7-P0, T7-P1 and T7-P4 with an empty vector, HA-MAT-Ubz1 or the double SprT mutant H111L/E112A and I blotted the lysates with the anti- γ H2AX antibody. As shown in Figure 4.21 (panel A), I detected a strong

band of γ H2AX when T7-P0 or T7-P1 was co-transfected with the empty vector (lane 2 and 3), whereas the intensity of that band decreased when T7-P0 or T7-P1 was co-transfected with HA-MAT-Ubz1 WT (lane 5 and 6). Moreover, γ H2AX was detected as a weak band when T7-P4 was co-transfected with the empty vector, which could only be seen in a long exposure (not shown) and the intensity of the band decreased when T7-P4 was co-transfected with HA-MAT-Ubz1 WT (lane 7 and long exposure, not shown). Importantly, the intensity of the γ H2AX band increased when the three T7-PCNA constructs were co-expressed with HA-MAT-Ubz1 (H111L/E112A), as shown in lanes 8-10 and in particular it was very high in the case of T7-P1, almost reaching the same levels as the HEK293T cells treated with UV that I used as a positive control (lane 11). These data indicate that the SprT domain suppresses the DNA damage signal caused by P1 expression. In order to check the level of γ H2AX when HA-MAT-Ubz1 was expressed alone, I transfected HA-MAT-Ubz1 WT, D473A and H111L/E112A in HEK293T cells and I blotted the lysates with the anti- γ H2AX antibody. As shown in Figure 4.21, the intensity of the bands detected in the HA-MAT-Ubz1 WT (lane 2) and H111L/E112A (lane 4) were quite similar, whereas the band detected for UBZ domain mutant (lane 3) was weaker. These results showed discrepancies with the results shown in figure 4.21 (panel A). For example Ubz1 WT appeared to decrease the level of the signal for DNA damage induced by P1 expression, we would expect that the intensity of the band detected for WT was weaker than in the SprT double mutant. To further investigate this difference, additional experiments using other DNA damage marker need to be performed.

A



B

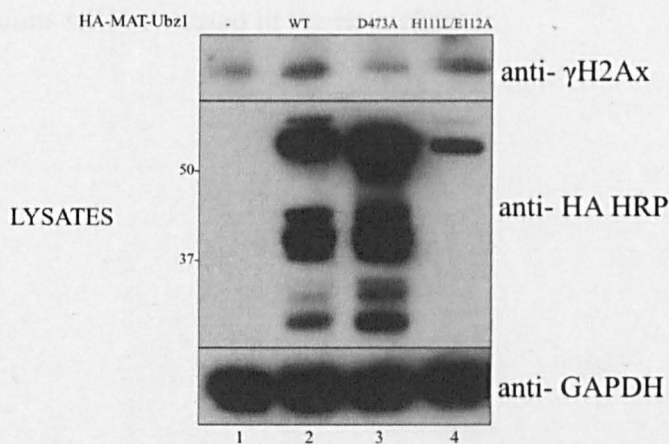


Figure 4.21. Level of γ H2AX upon T7-PCNA constructs and HA-MAT-Ubz1 co-transfection. Panel A: T7-P0, T7-P1 and T7-P4 were co-transfected with the empty vector, HA-MAT-Ubz1 WT or H111L/E112A mutant. Lysates were blotted with the anti- γ H2AX antibody (first panel) and with anti-HA-HRP and anti-T7 HRP antibodies to check the expression of HA-MAT-Ubz1 and T7-PCNA, respectively. The asterisk indicates the lower part of the anti-T7 HRP blot that shows the T7-Ub cleaved from T7-Ub-PCNA (T7-P1). Anti-GAPDH was used as a loading control. Panel B: HA-MAT-Ubz1 WT, D473A and H111L/E112A were expressed in the HEK293T cells and lysates were blotted with the anti- γ H2AX antibody. Anti-HA HRP was used to check HA-MAT-Ubz1 constructs expression and anti-GAPDH was used as a loading control. 2SPRT: H111L/E112A.

4.6. Conclusions

In my thesis work, I have shown that Ubz1 is a novel protein of UBZ family and that the HExH motif in its SprT domain might act as a metalloprotease. In particular, I have shown that Ubz1 binds unmodified and modified PCNA and the modified form is predicted to be PCNA ubiquitinated at the Lysine 164. The three PCNA fusion proteins that I built, allowed me to determine that during the co-purification of Ubz1 with the tagged-ubiquitin-PCNA fusion proteins, the mono-ubiquitinated fusion protein was cleaved in two parts, giving rise to tagged ubiquitin and free PCNA, and this appeared to happen only to the tagged-ubiquitin-PCNA that interacts with Ubz1. Moreover, I have shown that Ubz1 appeared to cleave itself in an SprT domain-dependent fashion, as the SprT mutant no longer showed degradation bands. The same SprT mutant also had a different effect on tagged-ubiquitin-PCNA expression and when they were co-purified, the PCNA fusion protein was no longer cleaved but appeared as a smear of PCNA. More detailed conclusions will be treated in the next chapter.

5. DISCUSSION

DNA damage repair is an important cellular pathway that must be regulated properly in order for chromosomal integrity to be maintained. The proliferating cell nuclear antigen, PCNA, is critical for the regulation of these repair pathways. PCNA tethers replicative polymerases onto DNA, thereby acting as a sliding clamp. In addition to tethering replicative polymerases, PCNA also recruits numerous other proteins, including Ubz1, to the replication fork and to sites of DNA damage repair.

During the S phase of the cell cycle, the genome is especially vulnerable and many kinds of lesions hamper replication fork progression. If the DNA replication does not restart, the replication fork collapses, leading to DNA double strand breaks and genetic instability. To prevent this, cells have evolved a particular pathway known as the DNA damage tolerance pathway, which allows the replication of the DNA past the lesion. The DNA damage tolerance pathway is error prone since the damage is left behind, to be repaired at a later time. In particular, when the replication fork encounters DNA damage, the replication fork stalls and PCNA can be mono-ubiquitinated at a single residue, K164, leading to an error prone pathway (translesion synthesis), or poly-ubiquitinated with a K63 linked poly-ubiquitin chain. Poly-ubiquitination of PCNA results in the use of a more error free pathway (Hoege et al., 2002). Ubz1 contains a PIP box and an ubiquitin binding domain, so it is strongly recruited to ubiquitinated PCNA.

5.1. Ubz1 is a Novel UBZ Family Protein

Previously, my lab attempted to identify effectors of the ubiquitin ligase BRCA1, a gene that is mutated in most inherited forms of breast cancer. BRCA1, a protein that is also

involved in DNA repair, produces a chain of ubiquitin molecules linked via Lysine number 6 (K6) (Wu-Baer et al., 2003). In a proteomics screen to identify effectors of BRCA1, my lab analyzed proteins that bind and/or are modified by K6 poly-ubiquitin chains and detected WHIP, whose yeast homolog has previously been annotated as functioning in DNA damage repair (Hishida et al., 2001). Further studies on WHIP, revealed that it contains an ubiquitin binding domain, annotated as Ubiquitin Binding Zinc finger domain or UBZ domain (Bish and Myers, 2007). Interestingly, the UBZ domain appears to be specific to the DNA replication and repair process, as all UBZ domain-containing proteins have been annotated to play this role in the cell. UBZ domains have been hypothesized to coordinate DNA repair activities by recruiting UBZ family proteins to the replication fork through an interaction with ubiquitinated PCNA or other ubiquitinated proteins at the point of DNA damage. The UBZ domain therefore appears have a specialized role in DNA repair, as opposed to a generalized ubiquitin binding function. Searching a sequence database for the proteins that contain a UBZ domain, my group also identified the protein C1ORF124 as a novel UBZ domain containing protein, which was called Ubz1. Ubz1 is a 55 kDa protein with three annotated domains: a PCNA interacting motif, an SprT domain and a UBZ domain (Centore, Yazinski et al. 2012). The function of the SprT domain is unknown. It carries a conserved metalloprotease HEXH motif, but no eukaryotic SprT domain proteins have been characterized.

In addition to their UBDs, a number of proteins involved in DNA damage repair pathway interact with PCNA through a second ubiquitin-independent contact, which presumably increases the affinity and specificity for the binding. As Ubz1 also contain a highly conserved PIP box motif and a UBZ domain, I thought that Ubz1 also might be involved in DNA damage response.

I have investigated various aspects of Ubz1 as the main focus of my thesis studies. I

started my studies to determine if Ubz1, as has been reported for other UBZ domain containing proteins, is subjected to a UBZ domain-dependent ubiquitination, known as coupled ubiquitination (Bish et al., 2008; Hoeller et al., 2006). Coupled mono-ubiquitination may have an inhibitory effect, resulting in the intra-molecular interaction between the UBZ domain and ubiquitin. As a matter of fact, mono-ubiquitination of POL η , in the absence of DNA damage, inhibits the interaction between POL η and PCNA during replication. Upon UV damage, PCNA is ubiquitinated and POL η is de-ubiquitinated. POL η is now in an open conformation and can bind PCNA (Bienko et al., 2010).

In my thesis work, I demonstrated that, Ubz1 is ubiquitinated. To characterize this post-translational modification, I performed a Denaturing-Tandem Affinity Purification (DTAP), which consists of a non-denaturing immunoprecipitation, followed by a denaturing purification; in this way, I detect only covalently attached ubiquitin. In fact, Ubz1 appeared as a smear, whereas the UBZ domain mutant ran as a single, non-ubiquitinated band (Figure 4.2). I initially tried to characterize the Ubz1 ubiquitination with anti-ubiquitin antibodies, but unfortunately I only detected the heavy and light chains of the antibody, but not the smear of ubiquitinated Ubz1. The experiment was repeated several times changing the conditions, but all my attempts failed and it is unclear why these experiments did not work. One possibility is that the detection of ubiquitinated proteins can be difficult depending on the amount and type of ubiquitin chain that is formed (Swerdlow et al., 1986). Since the analysis of proteins by mass spectrometry allows for the direct observation of post-translational modifications, I decided to make use of LC-MS/MS to confirm that the smear in the anti-HA blots of Figure 4.2 was caused by ubiquitination. Comparing the total number of peptides obtained from the mass-spectrometry analysis, I found a higher number of ubiquitinated peptides in the Ubz1 WT immunopurification than in the UBZ domain mutant (Table 4.1). In this way, I was able to

prove that Ubz1 is ubiquitinated and that this ubiquitination is dependent on its UBZ domain. In particular, mass spectrometry of these samples resulted in the identification of four Lysine residues that undergo ubiquitination (Figure 4.4) and all four sites are located between the PIP box motif and the UBZ domain of Ubz1 sequence. I thought that this particular arrangement might influence the binding capacity of the UBZ domain and the PIP box, but further experiments need to be performed in order to prove this. These results were further strengthened when I analyzed the immunopurified and peptide-eluted Ubz1 WT and UBZ mutant, with the α -ubiquitin antibody. In this way, I was finally able to detect a smear of ubiquitinated Ubz1 as shown in Figure 4.3.

My lab previously demonstrated that WHIP is subjected to a UBZ-dependent covalent poly-ubiquitination (Bish et al., 2008). The Ubz1 smear could correspond to the multiply-mono-ubiquitinated or to a poly-ubiquitinated form of Ubz1; since I found ubiquitin peptides corresponding to K48 in the Ubz1 WT sample, I thought that the smear might correspond to K48 linked poly-ubiquitinated Ubz1 rather than to multiply-mono-ubiquitinated Ubz1. My hypothesis was supported by the fact that more Ubz1 was ubiquitinated upon treatment with the proteasome inhibitor, as shown in Figure 4.5. Therefore the evidence indicates that Ubz1 undergoes coupled poly-ubiquitination and this coupled ubiquitination requires a functional UBZ domain. In this regard, the coupled ubiquitination of Ubz1 is similar to that observed for other UBZ domain containing proteins, such as WHIP. These results were quite consistent with the data recently reported, in which demonstrated that when Ubz1 (Spartan) was purified in denaturing conditions, it ran as a doublet with the upper band corresponding to ubiquitinated Ubz1 (Centore et al., 2012).

These data demonstrate that Ubz1 possesses a functional UBZ domain. As a matter of fact, I showed that the substitution of the Aspartate residue in the UBZ domain of Ubz1

is sufficient to reduce the ubiquitin binding of Ubz1. In fact, as previously discussed, mass spectrometry analysis revealed a larger number of ubiquitin peptides in the Ubz1 WT purification than in the UBZ mutant (34 versus 5 total peptides), Table 4.1. Therefore, my data demonstrated that the UBZ domain mutant no longer interact with ubiquitin. Based on this evidence I established that Ubz1 is a novel UBZ domain containing protein and that its UBZ domain binds ubiquitin.

5.2. A Physical Interaction Between Ubz1 and PCNA

The hypothesis that UBZ family proteins are recruited to sites of DNA damage by binding ubiquitinated PCNA is strengthened by the observation that most of these proteins also physically interact with PCNA. Three different peptide sequences have been found that permit other proteins to interact with PCNA. Many proteins that possess a UBZ domain, including POL η and POL κ , bind PCNA through their PIP box. Ubz1 contains an 8 amino acids long PIP box (325-332 residues), close to the C-terminal end of the protein sequence (Figure 5.1).

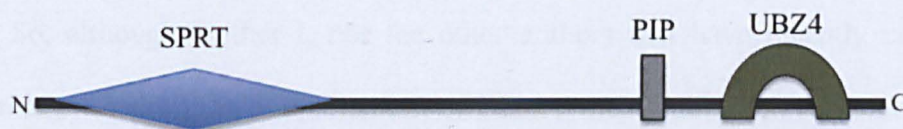


Figure 5.1. Cartoon of Ubz1. The N-terminal SprT domain is represented in blue, C-terminal UBZ4 in green and the PIP box in grey.

As part of my thesis, I demonstrated that PCNA interacts with Ubz1. Moreover, a second, higher molecular weight PCNA band also immunoprecipitated with Ubz1. This band was consistent with the predicted molecular weight of mono-ubiquitinated PCNA. Although PCNA is ubiquitinated upon DNA damage, it has been reported that a portion of

PCNA is constitutively mono-ubiquitinated even in absence of DNA damage in different cell lines, including the HEK293T cells that I used in my experiments (Chiu et al., 2006). Unfortunately, with the anti-ubiquitin antibody I was not able to confirm that that upper band of PCNA corresponded to its ubiquitinated form, but when I digested the immunoprecipitated HA-MAT-Ubz1 WT with trypsin and I analysed the sample by mass spectrometry, I found that PCNA was ubiquitinated at K164. Moreover, the finding that the UBZ mutant did not immunoprecipitate the upper band, suggested that it might be ubiquitinated PCNA. My data were consistent with the results reported by Zou's group, which shown that Ubz1 interacts with both the unmodified and more weakly with a modified form of PCNA, even in the absence of DNA damage (Centore et al., 2012). Moreover, they built a PIP box mutant, which when compared to the Ubz1 WT showed a decreased interaction with the unmodified PCNA, but not with its modified form, since it has an intact UBZ domain (Centore et al., 2012).

To gain further insights into the dependence of the interaction of Ubz1 with mono-ubiquitinated PCNA, I also purified the Ubz1 SprT double mutant and I have shown by LC-MS/MS that PCNA was not ubiquitinated at residue K164, as happened in the case of the WT. So, although neither I, nor the other authors that have recently characterized Ubz1, directly detected the ubiquitination of PCNA with α -ubiquitin antibodies, my data suggested that the upper band of PCNA is the ubiquitinated form of PCNA at K164.

5.3. A Possible Role for SprT Domain

The SprT domain (amino acids 45-212) of Ubz1 has an unknown function, but since it posses a HExH motif I thought that it might function as a metalloprotease. In the HExH motif, the two Histidine residues are ligands of a zinc atom, and the Glutamate residue has a catalytic role. The fourth ligand is a molecule of water that when activated,

mediates the nucleophilic attack on the peptide bond.

In my thesis work, I provided evidence that SprT might be a functional metalloprotease. These results were obtained using western blot analysis (Figures 4.8, 4.13, 4.14) and mass spectrometry (Figures 4.7, 4.15, 4.17, 4.18). First, I have shown that Ubz1 degradation requires its own SprT domain. As a matter of fact, Ubz1 ran in western blots as multiple bands and mass spectrometry analysis revealed that the band around 55 kDa contained peptides that covered the entire sequence of Ubz1 but surprisingly, the lowest portion of the gel contained peptides from the C-terminal end of Ubz1. Since Ubz1 had a HA tag at the N-terminal, the fact that I found peptides that belonged to the opposite terminus of the protein indicated that Ubz1 might cleave itself during purification, as shown in Figure 5.2. This hypothesis was strengthened by further western blot experiments that I performed on Ubz1 where I mutated the Histidine and the Glutamate of the SprT domain at positions 111 and 112, respectively. In fact, the SprT domain with a single mutant residue showed a decrease in the number of degradation bands, whereas the double mutant had virtually no degradation, shown in Figure 4.8. Therefore, the SprT domain is likely to be mediator of the Ubz1 cleavage, as shown in Figure 5.2.

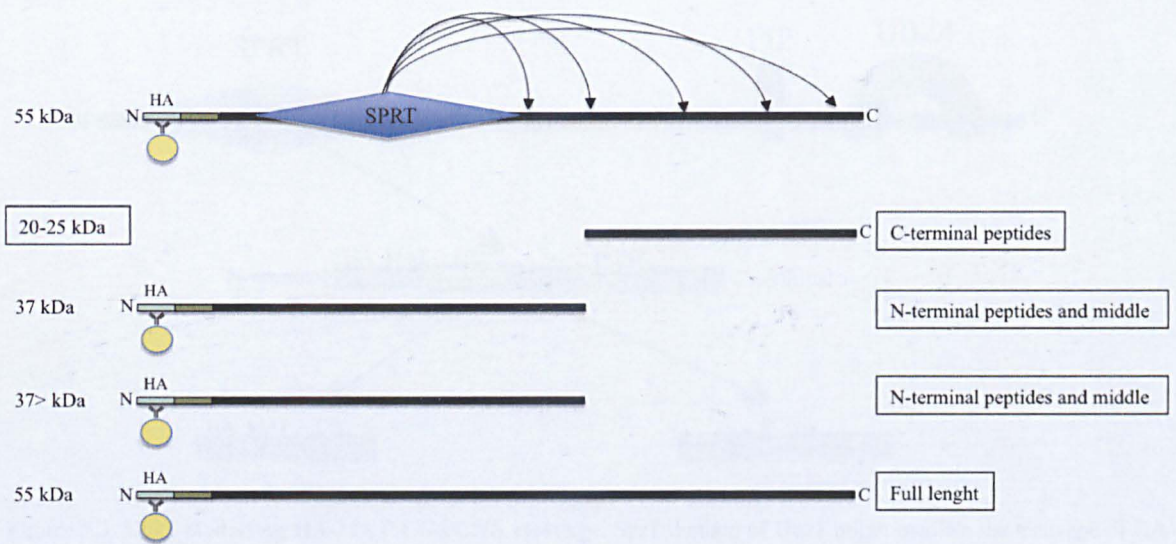


Figure 5.2. SprT domain mediating Ubz1 autocleavage. The cartoon indicates that the SprT domain of Ubz1 appears to mediate the Ubz1 autocleavage. The HA-MAT-Ubz1 bands between 37 and 50 kDa showed a large amount of peptides that covered the N-terminal portion of the protein, instead the band around 20-25 kDa surprisingly contained peptides coming from the C-terminal of HA-MAT-Ubz1, indicating that the SprT domain might act as a metalloprotease.

Furthermore, the SprT domain also plays a role in the interaction between Ubz1 and the ubiquitin-PCNA fusion protein. In fact, I showed that when the three PCNA fusion proteins tag-PCNA (P0), tag-Ub-PCNA (P1) and tag-4Ub-PCNA (P4) were co-expressed with Ubz1, P1 was cleaved giving rise to the tagged-ubiquitin and “free” PCNA, as seen in Figure 5.3.

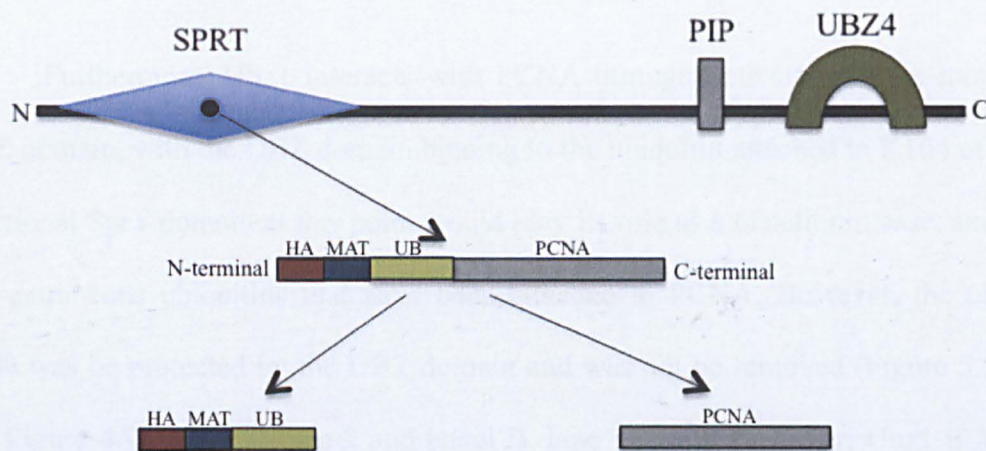


Figure 5.3. Ubz1 mediating HA-MAT-Ub-PCNA cleavage. SprT domain of Ubz1 might mediate the cleavage of HA-MAT-Ub-PCNA, giving the HA-MAT-Ub and the PCNA.

Given the fact that mutating the putative metalloprotease signature of the SprT domain blocks both the cleavage of Ubz1 and of P1, I believe that my data indicate that the SprT domain functions as a metalloprotease and the possible role of this domain during Ubz1/PCNA expression and purification is illustrated in Figure 5.4. In this case, when the SprT double mutant was co-expressed with P1 and the SprT domain was not able to cleave any of the ubiquitin molecules bound to PCNA, PCNA remained poly-ubiquitinated (Figure 5.4) and appeared as a high molecular weight smear in the blot, Figure 4.19. In this manner Ubz1 was acting as a de-ubiquitinase.

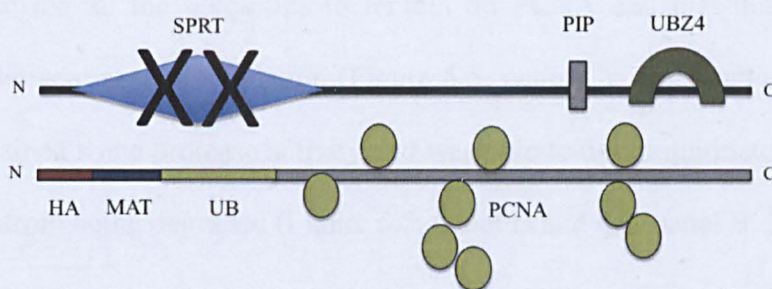


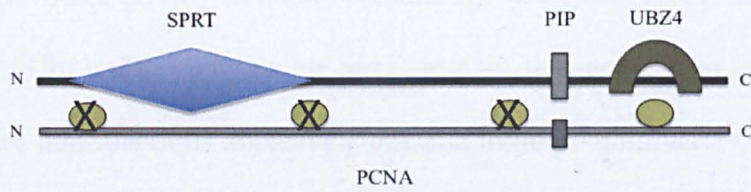
Figure 5.4. HA-MAT-Ub-PCNA poly-ubiquitination with Ubz1 SprT double mutant. The cartoon indicates that when the SprT domain of Ubz1 is mutated and no longer able to remove the ubiquitin bound to the HA-MAT-Ub-PCNA, HA-MAT-Ub-PCNA remains poly-ubiquitinated.

Furthermore, Ubz1 interacts with PCNA through both its PIP box motif and the UBZ domain, with the UBZ domain binding to the ubiquitin attached to K164 of PCNA. A functional SprT domain at this point would play its role as a metalloprotease, and removed any extraneous ubiquitins that have been attached to PCNA. However, the ubiquitin at K164 was be protected by the UBZ domain and was not be removed (Figure 5.5, panel A and Figure 4.9, panel A, lane 2 and panel B, lane 1). In this manner, Ubz1 is acting as a fidelity factor for PCNA ubiquitination. Most likely, the PIP box also plays a role in directing the binding of the UBZ domain of Ubz1 to K164 of PCNA.

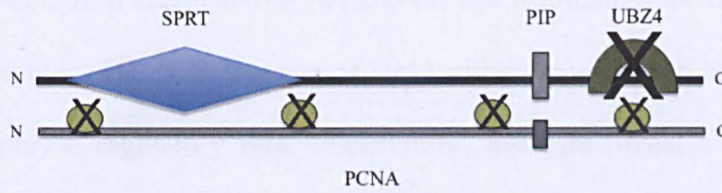
In the case where the UBZ domain mutant was expressed, Ubz1 interacted with PCNA only through its PIP box motif. The SprT domain was able to cleave all the ubiquitin molecules bound to PCNA, including those at Lysine 164 of PCNA (Figure 5.5, panel B). As a matter of fact, in the western blots I saw only one band for PCNA that immunoprecipitated with Ubz1, which corresponds to the unmodified form of PCNA (Figure 4.9, panel B, lane 2).

In the case of the SprT double mutant, Ubz1 was no longer able to act as a metalloprotease and I did not detect any band of PCNA co-immunoprecipitating with Ubz1 (Figure 4.9, panel A, lane 3 and panel B, lane 4). I proposed that the non-functional SprT domain allowed all the ubiquitins to remain on PCNA and that this poly-ubiquitinated PCNA underwent rapid degradation (Figure 5.5, panel C). The single mutant in the SprT domain retained some protease activity and was able to de-ubiquitinate a portion of PCNA and kept it from being degraded (Figure 5.5, panel D and 4.9, panel B, lane 3).

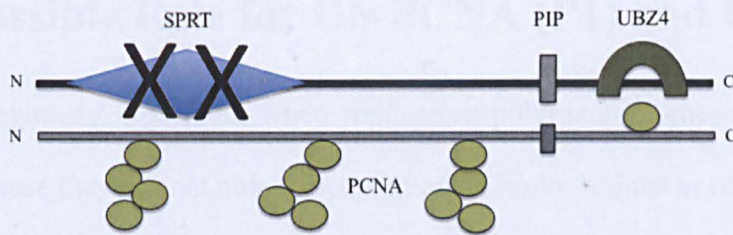
A



B



C



D

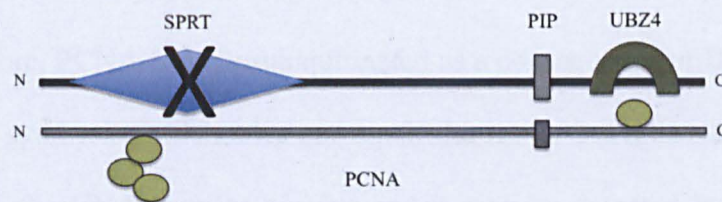


Figure 5.5. Model proposed for SprT domain role. Panel A: Ubz1 WT. PIP box binds PCNA. The UBZ domain protects the ubiquitin bound to K164 of PCNA from SprT cleavage. Panel B: Ubz1 UBZ mutant. PIP box binds the PCNA. The UBZ domain cannot protect ubiquitin bound to K164 of PCNA from SprT cleavage. Panel C: Ubz1 SprT domain double mutant. PIP box binds the PCNA. SprT domain cannot cleave ubiquitins bound to the PCNA. Panel D. Ubz1 SprT single mutant. PIP box binds the PCNA. SprT domain partially cleaves ubiquitins bound to the PCNA.

In summary, my thesis work indicates that Ubz1 contains a functional UBZ domain and that like other UBZ domain containing proteins, Ubz1 undergoes coupled ubiquitination. Ubz1 also contains an SprT domain that appears to encode a functional metalloprotease that functions to cleave Ubz1 and to de-ubiquitinate PCNA. Interestingly, the UBZ domain and the SprT domain function together as a fidelity factor, which results in PCNA being ubiquitinated only at K164. This is due to the UBZ domain binding and protecting the ubiquitin attached to K164 and the SprT domain removing the other attached ubiquitin molecules. The autocleavage of Ubz1 either occurs as an artefact of purification or may also play a regulatory role. Importantly, my data reveal a previously unknown mechanism regulating the ubiquitination of PCNA.

5.4. A Possible Role for Ub-PCNA (P1) and Ubz1

As previously described, when replicative polymerases encounter DNA damage, they stall because they are not able to accommodate bulky lesions or mismatched bases. As a consequence, PCNA is mono-ubiquitinated and the translesion polymerases (TLS POL), which have a major affinity for mono-ubiquitinated PCNA, replace the replicative polymerases. Once the TLS POL replicate past the damage, PCNA is de-ubiquitinated by USP1. Therefore, PCNA is mono-ubiquitinated as a consequence of DNA damage, leading to translesion synthesis, which does not repair the lesion but rather bypasses the damage. Consequently, the DNA damage persists and it may be detected by the cell machinery, which results in the activation of the ATM/ATR signalling pathway. ATM/ATR phosphorylate different substrates, including CHK1, CHK2 and γ H2AX. This pathway might be an explanation of why P1 expression increases the level of γ H2AX, as shown in Figure 4.12. Another possibility might be that P1 is being considered as a signal of damage

itself, even in absence of any real DNA damage and as a consequence P1 activates ATM/ATR signalling, resulting in the increased levels of γ H2AX.

Ubz1 appears to be functionally linked to PCNA. In fact, it has been recently reported that Ubz1 is principally expressed during S and G2 phase of the cell cycle (Mosbech et al., 2012), which is also the phase of the cell cycle where PCNA mainly exerts its function. Moreover, my preliminary data obtained from cyclohexamide experiments, suggested that Ubz1 is present in two different pools in the cell, one undergoing rapid degradation and one undergoing slower degradation. The blot in Figure 4.20 shows that only the full-length Ubz1, but not the degradation products, immunoprecipitated with the Ub-PCNA (P1) suggesting that PCNA may be a stabilization factor for Ubz1. In this way, the pool of Ubz1 that was rapidly degraded would represent the free pool of Ubz1, whereas the Ubz1 that was slowly degraded, would be the pool bound to PCNA.

Ubz1 also appeared to act as a de-ubiquitinase. It has been reported that the USP1 de-ubiquitinase autocleaves itself upon UV damage, recognizing an ubiquitin-like di-glycine (GG) motif in its own sequence (Huang et al., 2006). The initial goal of the analysis of Ubz1 degradation bands had been to detect if the final amino acids of the peptides contained in the lower bands matched with the di-glycine (GG) motif, but that was not the case. Since if a lesion is not repaired during the S phase of the cell cycle, the replication fork may collapse leading to a double strand break, cells have developed the TLS mechanism in order to replicate past the damage. Mono-ubiquitinated PCNA has a key role in recruiting TLS polymerases to the site of the stalled fork and my data suggested that Ubz1 appeared to ensure PCNA mono-ubiquitination at the K164. In particular, the PIP box and the UBZ domain of Ubz1 are necessary for a correct positioning of the protein for its interaction with the mono-ubiquitinated PCNA (Figure 5.5, panel A) and the SprT

and the UBZ domain together collaborate to maintain the PCNA being mono-ubiquitinated specifically at K164. As a matter of fact, the SprT domain cleaves all the ubiquitin bound to the PCNA except at K164, which is protected by the UBZ domain. These data were consistent with recent results in which it was demonstrated that upon Ubz1 knockdown, in case of UV, there was a decrease of the recruitment of polymerase η at the site of the damage (Centore et al., 2012). Thus, Ubz1 appears to be important for the recruitment of TLS polymerase at the site of the stalled fork, promoting a damage bypass and avoiding the collapse of the replication fork with more dramatic consequences.

Recently, several labs have characterized Ubz1. In particular, Ubz1 has been found to be involved in single strand DNA damage response (Centore et al., 2012; Davis et al., 2012; Ghosal et al., 2012; Juhasz et al., 2012; Machida et al., 2012; Mosbech et al., 2012). The UBZ domain was found to be important for the localization of Ubz1 to the sites of DNA damage, but there are some discrepancies in this regard. For example, it is not clear if the mono-ubiquitination of PCNA is dispensable for the recruitment of Ubz1 to the sites of the DNA damage. In this regard, upon the UV treatment, even in case of knockout of Rad18, Ubz1 appeared to be recruited to the damage foci. Two groups hypothesized that Ubz1 is recruited to the sites of damage by other ubiquitinated factors (Davis et al., 2012, Mosbech et al., 2012). In addition, Ubz1 seems to have an effect on PCNA mono-ubiquitination, but the data in this case are not in agreement. In particular, upon Ubz1 knockdown, following UV damage, mono-ubiquitination of PCNA decreases, leading to a minor recruitment of polymerase η at the sites of damage (Centore et al., 2012), but these data are not consistent with two more recent papers that report that following UV damage and Ubz1 knockdown, the PCNA mono-ubiquitination increases and there is a persistence of the TLS polymerases at the damage foci. Although no model proposed by the authors seems to be very consistent with my results, Juhasz et al. have recently reported that Ubz1

ensures the level of ubiquitination of PCNA and these data appeared to support my model proposed in Figure 5.5 (Juhasz et al., 2012). Although the role of the UBZ domain and PIP box of Ubx1 have been characterized, only one group has investigated the Sprt domain role. In particular, they show that the Sprt domain of Ubx1 appeared to be necessary for suppression of damage-induced mutagenesis, but this was only a minor experiment in the paper (Kim et al., 2013). Importantly, my work indicates that the SprT domain is a functional protease that works in concert with the UBZ domain to regulate the ubiquitination of PCNA.

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